UNIVERSITY OF EDINBURGH

INTERRELATIONSHIPS OF GRAM-NEGATIVE BACTERIA AND PLANT ROOTS.

by

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C O N T E N T S

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INTRODUCTION.

Introduction.

A review of the literature on soil microbiology shows that extensive studies have been made into the microbial activity which is associated with the roots of plants. However, in spite of this intensive study into the so-called "rhizosphere effect" only three generalised conclusions can be drawn concerning the bacteriological aspects of the subject. Even the validity of these has been doubted by some workers. Firstly, the growth of Gram-negative organisms is preferentially promoted; secondly, the organisms have greater physiological activity than non-rhizosphere organisms and thirdly, there is an increase in the percentage of organisms which require amino acids for maximum development.

One of the aspects of the subject which has been largely ignored and which would appear to be useful is knowledge of the combined physiological, biochemical and nutritional properties of the individual organisms in the rhizosphere. In particular the Gram-negative types are of interest since they apparently respond more than the other groups to the presence of the plant root. An attempt is made in this study to obtain a broad picture of the activities of the Gram-negative bacteria in the rhizosphere with the object of clarifying the reason for their stimulation, studying their possible rôle in the rhizosphere and of classifying the organisms so that comparison might be made with organisms which occur in other restricted environments.

Knowledge/

Knowledge is very limited on the effect which the plant environment has on the rhizosphere microflora. Experiments carried out in this study show how variation of the medium and climatic conditions in which the plant is growing effect the rhizosphere Gram-negative microflora.

Finally, there have been many investigations into the mutual effects which bacteria and plants have on each other whilst the plant is growing in soil. However there have been very few experiments with pure cultures. Experiments conducted in this investigation show the effect of pure and simple mixed cultures on plants growing under several different nutrient conditions.

REVIEW OF THE LITERATURE

Review of the Literature

The reviews of Norman (1946), Katznelson, Lochhead & Timonin (1948), Clark (1949) and Starkey (1958) have outlined the developments in the microbiology of the rhizosphere which have taken place since Hiltner (1904) first appreciated that the soil zone in the immediate vicinity ofthe root possessed intense microbiological activity.

For the purpose of this review, the literature has been divided into the four sections which are most pertinent to the field of study.

a) The occurrence of Gram-negative organisms in the rhizosphere.

Löhnis (1926) carried out some of the earliest experiments concerned with the effect of plant growth on the soil microflora. He showed that organisms in the Bacterium radiobacter group were more numerous in soil after leguminous crops had been grown than after non-leguminous crops. Smith (1928) estimated the numbers of B. radiobacter in soil by counting the raised glistening colonies which developed on glycerol nitrate agar after 1:20,000 crystal violet had been added to the dilution water. Smith noted that the growth of cereals reduced the numbers of B. radiobacter whereas several leguminous crops increased the count. It seems probable that Smith was counting other organisms/

organisms beside B. radiobacter and the increase in the case of the leguminous crops could have been Rhizobium species.

Starkey (1929a) showed on the roots of apple and alfalfa that organisms producing mucoid colonies on nitrogen-free mannitol agar increased more rapidly than any other group which he studied. He regarded the organisms producing these mucoid colonies as belonging to the Bacterium radiobacter - Aerobacter aerogenes - Bacillus radicicola group. In later experiments Starkey (1929b) examined several plants for mucoid colony-producing organisms. Oat and rape gave the largest increase in the early stages, but sweet clover was the only plant which did not show a reduction in later periods of plant development. Starkey concluded that bacteria were affected more than any other microbiological group which he examined.

Clark (1940) made total counts of control soil and rhizosphere soil on egg-albumin agar and counted the dye-tolerant bacteria which developed on the same medium containing crystal violet at a concentration of 1:100,000. In addition Clark also counted blue-green fluorescent bacteria which developed in asparagine mineral salts solution. In experiments with the cotton plant he found that the greatest number of organisms were on root surface scrapings where also a larger percentage of Gram-negative bacteria were to

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be found. Both total numbers and the percentage of Gram-negative organisms decreased with the distance from the root. Examination of 400 isolates from the root scrapings showed that 67% of the organisms were Gram-negative rods compared with 20% from the soil a distance from the root. Forty percent of the control soil isolates and 12% of the root scrapings isolates were Gram-variable or Gram-positive rods which Clark considered to be in the Corynebacterium group. Less than 1% of the total isolates produced a blue-green fluorescent pigment and rarely more than 5% of the isolates were Bacterium radiobacter.

In the examination of organisms from wheat roots Clark found that the majority of 600 isolates were coccoidal in older culture and were probably in the coryneform group. The percentage of dye-tolerant types only increased slightly. Specific Gram-negative groups seldom comprised more than 10%. Preliminary studies of the blue-green fluorescent Pseudomonas types showed that the majority of the root isolates utilised sucrose and starch whereas the soil isolates were unable to utilise these substances. In further examinations of the roots of different plants Clark found that the percentage of strains of Pseudomonas present varied with the type of plant studied. He found 1.7% on wheat, 3.7% on barley, 7.35% on corn and 29.8% on radishes.

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In the same year Lochhead (1940) examined the effect of plant growth on the characteristics of the overall bacterial population of a wide variety of plants. He found that Gram-negative rods were proportionately increased, and that there was a higher proportion of motile organisms, chromogenic types, organisms liquefying gelatin and of those affecting glucose. Lochhead found that the large majority of the organisms showed an affinity to corynebacteria being pleomorphic on Topping's medium. True Gram-negative rods which were not pleomorphic varied from under 10% to usually about 20%. Lochhead made no attempt to characterise or classify the individual organisms.

A detailed examination of the types of organisms occurring in the rhizosphere of the pineapple was carried out by Contois (1953). He characterised and classified his isolates on the basis of Bergey's Manual (1948), but no data were given as to the relative abundance of the many specific types which he identified.

Webley, Eastwood & Gimingham (1952) examined the development of the soil microflora in relation to plant succession in sand dunes. The rhizosphere bacterial count increased as the plant flora became more complex. Some plants gave higher rhizosphere counts than others. The development of Agropyron junceum did not preferentially promote/

promote in the rhizosphere the growth of Gram-negative bacteria.

After an investigation of the rhizosphere of graminaceous plants growing under conditions prevailing in virgin soils, Gyllenberg (1955) concluded that the number of bacteria in the rhizosphere was between 11 and 31 times as great as that in the control soil. She found the effect of plant growth on non-sporogenous bacteria to be constant irrespective of the plant species, stage of plant development and soil conditions. The Gram-negative bacteria were more abundant than the Gram-positive types. She considered that the Gram-positive organisms were mainly typical coryneform organisms and that the Gram-negative types were in the genera Achromobacter, Flavobacterium and Pseudomonas. Gyllenberg did not give her reasons for placing the organisms in these genera.

Rovira (1956a) made a slightly different approach to the problem. He germinated seeds of oats and tomato on glass beads and in sand and found that Gram-negative chromogenic types from the seed multiplied rapidly on the oat seedlings but not on the tomato seedlings. Rovira (1956d) showed that separated root exudates, added to sand, preferentially promoted the growth of Gram-negative organisms. This led Rovira to conclude that conditions similar to those found

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in the rhizosphere were being produced.

Abstracts of rhizosphere work done recently in Eastern Europe have indicated that in general the emphasis has been on the development of Gram-negative organisms. Nette (1955) found an increase in the number of organisms in the genera Achromobacter and Pseudomonas in the rhizosphere of oak and Kozlova (1955) cited cases where 50% of the rhizosphere microflora of old oak trees comprised members of the genus Chromobacterium. Strains of Pseudomonas also formed an appreciable part of the flora.

This review of the literature emphasises the fragmentary nature of the present information available on the ecology of the Gram-negative organisms in the rhizosphere. It can be seen that no comprehensive investigation of the rhizosphere bacterial microflora has yet been attempted.

b) The biochemical and physiological characteristics of the Gram-negative bacteria of the rhizosphere.

Many of the experiments concerned with the physiological characteristics of the entire rhizosphere microflora have been carried out in Ottawa by Lochhead, Katznelson and their co-workers. As early as 1940 Lochhead, Timonin & West (1940) noted that a higher percentage of organisms requiring amino acids were isolated from the rhizosphere of flax and tobacco than from the control soil. The large amount of work carried/

carried out since that time on nutritional groups of bacteria occurring in the rhizosphere, in particular the growth of amino acid-requiring types has been discussed by Starkey in his review. This stimulation of amino acid-requiring organisms has led the observers to conclude that amino acids are excreted by the experimental plants in amounts which significantly effect the rhizosphere microflora. This point will be discussed later, but it is of interest to note that Lochhead & Thexton (1947), Gyllenberg (1956) and Payne, Rouatt & Lochhead (1957) have shown that the filtrates of isolates which do not require amino acids for maximum development, are capable of supporting the growth of the organisms which do have this nutritional requirement.

More recent work confirming the Ottawa findings has been done by Gyllenberg (1957). She found that the preferential stimulation of amino acid-requiring organisms persisted for the whole growing season and that the soil flora between rows of oat plants gradually became similar in composition to the rhizosphere population.

However, Wallace & King (1954) were unable to demonstrate the preferential growth of organisms requiring amino acids in the rhizosphere of barley and oats.

Rovira (1956c) came to the conclusion that the excretion of growth factors was more important than that of amino acids./

acids. He found that the exudate of peas and oats gave a similar rhizosphere effect even though the pea roots excreted larger quantities of amino acids. Rovira also felt that there was no plant specifity in the response of the rhizosphere microflora. The organisms which are capable of the most rapid multiplication develop in the rhizosphere.

Following up this point several workers have compared the physiological activity of rhizosphere and control soil isolates. In general it has been shown that the rhizosphere isolates are far more active physiologically. Rouatt & Katznelson (1957) compared the growth of the two groups of isolates on five different media. The rhizosphere isolates of all the plants tested except oats grew better than the soil isolates. The oat rhizosphere isolates grew to approximately the same extent as the control soil isolates. Similarly Katznelson & Rouatt (1957) showed that rhizosphere isolates were more active physiologically than the control soil isolates by testing their ability to reduce methylene blue and resazurin, produce acid and gas from glucose and their ammonifying and denitrifying capacity. Nette (1955) also found that denitrifying organisms were more numerous in the rhizosphere of oak than in the control.

King & Wallace (1956) observed a reduction in the physiological activities of the rhizosphere isolates compared with/

with the control soil organisms, except for the higher proportion of nitrate reducing bacteria in the rhizosphere of young oat plants.

In manometric studies Zagallo & Katznelson (1957) found the rhizosphere isolates were more active than the control soil isolates in the oxidation of glucose, alanine, acetate, possibly succinate, but not of pyruvate and xylose.

Gyllenberg (1956) examined 60 non-sporogenous bacteria isolated from the roots of graminaceous plants. She divided the organisms into three groups on the basis of their nutritional requirements. The organisms which grew with nitrate as the sole source of nitrogen were placed in the genus Pseudomonas. The group which required amino acids were placed in either the genus Achromobacter or Flavobacterium and those requiring both amino acids and B vitamins were allocated mainly to the genus Corynebacterium. Contrary to the results obtained by Lochhead (1940), Gyllenberg found that only a few of her isolates showed pronounced proteolytic activity.

Whilst comparisons have been made of certain physiological activities of isolates from rhizosphere and non-rhizosphere soils, little is known of the combined characteristics of any one group of organisms. Until these combined characteristics have been compared it will be difficult to obtain a better understanding/

understanding of factors affecting the development of organisms in the rhizosphere.

c) Organic materials liberated by plant roots in relation to the rhizosphere effect.

The experiments of Lyon & Wilson (1921) were amongst the earliest which demonstrated the liberation of organic matter by the roots of growing plants. When they grew maize, oats, peas and vetch in sterile nutrient solution containing nitrate, organic nitrogen appeared in the solution within several weeks and before all the nitrate nitrogen has been used up. Lyon & Wilson felt that more organic matter had appeared than could be accounted for by sloughed off cells alone. Only about 2% of the organic matter, which they estimated, was nitrogenous. Little work was done on the subject until West (1939) showed by microbiological assay procedures that thiamin and biotin were excreted from young roots of flax. West found that older roots excreted unknown inhibitory material which interfered with the growth of the Staphylococcus aureus which was being used for the assay of thiamin. West & Lochhead (1940) concluded that thiamin, biotin and amino nitrogen were excreted in significant amounts because they isolated rhizosphere organisms which were dependant on these substances for their development. The effect was shown even at the seedling stage, which led the authors to conclude that

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it was excretion and not decomposition which was causing the effect.

The emphasis on amino acid excretion was developed by Katznelson, Rouatt & Payne (1954) and (1956) when they demonstrated that 6 - 8 weeks old soybean, tomato and wheat plants absorbed through their leaves amino acids which could be recovered from the mineral solution in which the plants were growing. They also demonstrated that if a sand-soil mixture in which pea and wheat plants were growing was allowed to dry out and was then remoistened, a variety of amino acids and reducing compounds were liberated by the roots. The quantity of the compounds liberated was appreciably higher than that liberated by plants, whose root environments were not allowed to dry out. No attempt was made by these workers to differentiate between excreted material and "sloughed off" cells. Parkinson (1955) also demonstrated the excretion of amino acids from oat seedlings by perfusing nutrient solution through the sand in which they were growing. He identified cysteine, glycine, alanine and aspartic acid.

Rovira (1956b) reviewed the literature concerning the compounds excreted by plants. These included phosphatides, amino acids, vitamins, glucose, nucleotides, and unidentified toxic and non-toxic substances, but no mention was made of the/

the excretion of plant acids having been observed. Rovira examined the root exudate of peas and oats. In an amino acid estimation he separated 27 amino compounds, and he also obtained glucose and fructose when the plants were 10 days old but not at 21 days. He noted some differences between oats and peas.

Andal, Bhuvaneswari & Subba Rao (1956) examined the root exudate of varieties of rice, resistant and susceptible to Fusarium moniliforme, during the first week of growth. Differences were noted between the resistant and susceptible varieties.

Bhuvaneswari & Subba Rao (1957) analysed the root exudate of sorghum and mustard for certain sugars and organic acids. They identified tartaric acid, oxalic acid, D-xylose and D-fructose in the exudates of both plants. In addition malic acid, citric acid, D-glucose and maltose were detected in the exudate of mustard roots. These were the only experiments found in the literature where plant acids had been shown to be exuded by plant roots.

In the examination of root exudates the emphasis has been on amino acids. It appears however that a wide variety of other organic compounds can be excreted by certain plants under certain conditions. Very little seems to be known of the effect which the plant environment has on the excretion of these materials. No study has been made of the

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ways in which micro-organisms affect the exudation of the organic materials. In addition, the possibility that this organic material may be liberated from "sloughed off" cells has been rarely considered.

A rhizosphere process which has not been examined extensively is the adaptation of micro-organisms to the root exudate of specific plants. Metz (1955) examined the effect of root sap and fragments of root on rhizosphere organisms. The plant materials were not antagonistic to the plant's own rhizosphere microflora, but were antagonistic to the rhizosphere isolates from other plants. This could be either adaptation or selection. A more definite example of adaptation by Zinovera (1957) demonstrated the increased development of strains of Azotobacter in the rhizosphere of plants to which they had specifically been adapted. These examples are among the few known which indicate plant specificity in the rhizosphere population.

d) The effect of pure cultures of bacteria on growing plants.

Very few investigations have been made into the relationships between growing plants and pure cultures of bacteria. Some of the earliest work was carried out by Wilson & Lyon (1926). They showed that maize and timothy plants growing in soil stimulated the multiplication of pure cultures/

cultures of selected organisms. Later experiments by Steinberg (1947b) were conducted during an investigation into the cause of "frenching" in the tobacco plant. Steinberg grew tobacco seedlings in mineral agar containing 200 p.p.m. peptone. At a distance from the root, the agar was inoculated with pure cultures of non-pathogenic soil bacteria. Sixty of these organisms produced gross changes in the tobacco plant, including chlorosis and symptoms similar to mineral deficiencies. Morphological changes resembling "frenching" were also observed. Steinberg pointed out in this paper and also had demonstrated earlier (Steinberg 1947a), that 200 p.p.m. of dl-isoleucine induced "frenching" symptoms. It is possible that isoleucine was being released from the peptone by the bacteria. Steinberg (1951a) later showed that Bacillus cereus strains could produce "frenching" symptoms. He also showed that the rhizosphere of affected plants harboured appreciably higher numbers of Bacillus cereus than the control plants. However Jones & Tio (1948) showed that an application of $FeSO_4$ prevented "frenching" and concluded that biologically induced iron deficiency was the cause of the condition. The situation remains obscure.

More recent studies by Pántos (1956) and Akhromecko & Shestakova (1957) have produced rather conflicting evidence. Pántos/

Pántos inoculated sterile wheat seedlings, growing in sand culture, with aqueous suspensions of Pseudomonas radiobacter, Flavobacterium solare, Bacterium parvulum and B. agile. The organisms brought about an increase in dry matter production and total nitrogen content in the plant. Akhromecko & Shestakova showed that inoculation with pure cultures of unnamed organisms, of maple, oak and ash growing in sand-nutrient solution decreased the phosphorus uptake of the plants. In addition they showed that some organisms brought about increased excretion of sulphur and phosphorus compounds by the roots.

These fragmentary bits of evidence indicate that practically nothing is known of the effect of growing plants on the individual bacteria found in the rhizosphere. Similarly little is known of the influence which pure cultures of bacteria exert on plants growing under different environmental conditions.

MATERIALS AND METHODS

Materials and Methods.

A) Cultural experiments.

The cultures used in the experimental work were obtained by direct isolation from the rhizosphere at the time of harvesting the plant, or from the control soil before the seeds were sown. Seed isolates were obtained from the batch of seed which was to be used for the experimental work.

Method of isolation of the organisms.

1) Rhizosphere organisms. At the time of harvesting the plant from soil the root system was carefully removed from the ground or pot in which the plant had been growing. Taking care not to contaminate the root, the surplus soil was removed from the root system with sterile forceps and the upper part of the plant cut off at ground level. The root system was then placed in a sterile mortar, a little sterile tap water was added and the material was ground. This material was then poured into a dilution bottle whose contents were then made to 100 ml. with repeated washings of the mortar and pestle. After vigorous shaking the material was serially diluted in sterile tap water and immediately plated. For the isolation of organisms from roots grown in nutrient solution only a portion of the very dense root system was added to the mortar.

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2) Control soil organisms. Ten grams of fresh soil were added to the dilution bottle and the contents made up to 100 ml. with sterile tap water. This material was then vigorously shaken, diluted and plated.

3) Seed organisms. About ten seeds were macerated in a sterile mortar before dilution and plating.

Total counts of organisms.

In all the experiments 1 ml. of diluted material was plated with 15 ml. of soil extract agar (Cunningham 1947) to which 0.2%yeast extract (Difco) had been added prior to sterilisation. The plates containing this medium (S.E.Y.E.) were incubated at 22° for 14 days.

Count of Gram-negative organisms.

Holding (1954) found that of several media tested, nutrient agar containing 1:500,000 concentration of crystal violet (C.V. agar) gave the highest count of Gram-negative organisms. The medium contained peptone (Evans) 5 g.; lemco 5 g.; agar (N.Z. Davis) 15 g.; tap water 1 l.; pH 6.8. After melting and cooling to 50°, 1 ml. of sterile 1:5000 crystal violet was added immediately prior to pouring the plates. The plates were dried in a 37° incubator for about one hour before 0.1 ml. of the diluted suspension was pipetted onto the surface. The drop was spread over the entire/

entire surface of the plate, with a sterile glass rod. The plates were incubated at 22° for 7 days. After 2 days incubation the plates were examined under the ultra-violet lamp and colonies producing a fluorescent pigment were noted. With longer incubation periods diffusion of the pigment into the medium made accurate observation of the fluorescent colonies impossible. If the incubation time was extended beyond 7 days many small colonies developed which were usually Gram-positive or Gram-variable coryneform organisms. Absorption of the dye by the faster growing Gram-negative organisms was probably the reason for the late development of the Gram-positive types. Examination of the colonies at the end of the 7-day incubation period enabled some Gram-negative organisms to be identified. This aspect will be discussed under the section concerned with the classification of the organisms.

After this work had been completed Board (1958) reported that some Gram-negative bacteria are sensitive to the peroxide which is present in some laboratory media. This effect can be removed by adding blood or manganese dioxide to the medium. Accordingly, an experiment was devised to find out whether peroxide in C.V. agar was inhibiting any of the Gram-negative organisms found in soil. Serial dilutions of a garden soil were prepared and surface plated (0.1 ml.) onto/

Table 1. The effect of blood on the count of Gram-negative organisms developing on three media containing crystal violet.

	Medium	Counts (10^{-4})	Average count
1	Topping's	109 120 117	115
1a	Topping's + blood	128 107 107	114
2	C.V. agar	97 132 117	115
2a	C.V. agar + blood	99 99 99	99
3	S.E.Y.E.	133 141 147	140
3a	S.E.Y.E. + blood	139 135 145	140

onto the following media.

Medium 1) Yeastrel, 2.5 g.; peptone (Evans) 2.5 g.; agar (N.Z. Davis) 15 g.; tap water, 1 l.; pH 6.8 (Topping 1937) + 1:500,000 C.V.

Medium 2) C.V. agar.

Medium 3) S.E.Y.E. + 1:500,000 C.V.

Media 1a, 2a and 3a contained 1% laked blood in addition to the ingredients detailed above. The blood had been prepared by adding 5 ml. of fresh sheep blood to 10 ml. of sterile distilled water. The plates were inoculated in triplicate and incubated for 7-days at 22°. The results are shown in Table 1.

Gram staining of the colonies showed that the increase in count on the S.E.Y.E. media was due to the development of Gram-positive and Gram-variable organisms of coryneform morphology. In this experiment the addition of laked blood did not bring about an increase in the count. It was concluded that the constituents of the media tested did not possess toxic materials which were neutralised by the blood.

Maintenance of stock cultures.

Colonies developing on the C.V. agar plates were examined by a hand lens and representatives of each morphological and pigmented type were transferred to Topping's medium. After incubation/

incubation at 22° the cultures were purified by two platings on the same medium. Pure cultures were then transferred to the medium and stored at room temperature. The stock cultures were sub-cultured every three months.

The following examinations were made during the course of the study.

a) Gram reaction.

Hucker's (1927) modification of the Gram staining procedure was used. The cultures were inoculated onto Topping's medium and incubated at 22° for 18 - 24 hr. before staining.

b) Examination for motility.

Organisms were examined for motility by the hanging drop method after incubation at 22° for 18 - 24 hr. Gray's method (1926) was used for staining flagella.

c) Utilisation of sugars and plant acids.

The following basal medium was used: $NH_4H_2PO_4$, 0.5 g.; K_2HPO_4, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl, 5 g.; agar 15 g.; distilled water, 1 l.; yeast extract (Difco), 0.5 g.; pH 6.8. Inoculations in this and later experiments were carried out by adding to the medium one drop of a slightly turbid suspension of the organism in sterile tap water.

1) Oxidation and fermentation of glucose.

Bromo-cresol-purple, 0.01 g. was added to the basal medium/

medium prior to sterilisation. After sterilisation filter-sterilised glucose was added to give a final concentration of 0.5% glucose. Actively growing cultures were stab inoculated into 10 ml. of the medium in $\frac{5}{8}$ in. tubes. Cultures were examined daily for acid or acid and gas production. Growth and acid production at the top indicated a purely oxidative metabolism, whereas growth and acid production throughout (or acid and gas) indicated anaerobic growth and a fermentative metabolism. Reversion of the acid at the top appeared to have no significance in the aerobic groups but this action in the facultative anaerobes indicated an acid-neutralising mechanism similar to that found in Aerobacter aerogenes.

2) Acid production from carbohydrates.

Bromo-cresol-purple, 0.01 g. was added to the basal medium prior to sterilisation. After sterilisation the filter-sterilised carbohydrate was added to give a final concentration of 0.5% carbohydrate. Slope cultures were prepared using 5 ml. of the medium in $\frac{5}{8}$ in. tubes. Cultures were examined daily for acid production. Organisms which did not produce acid from glucose were reinoculated into the same medium with and without the added carbohydrate. Cultures which did not show any difference in the quantity of growth on the two media were assumed to be unable to utilise the glucose. Growth characteristics were more easily observed on sloped

agar/

agar than in stab inoculations. It was found that organisms which were unable to utilise glucose could not utilise other carbohydrates, but the converse was not always true.

3) Oxidation of plant acids.

Bromo-thymol-blue, 0.01 g.; and 5 g. of the Na salt of the plant acid were added to the basal medium. The pH was adjusted to 6.8. Indicator colour changes were compared with a control medium to which no salt of the plant acid had been added. A higher final pH in the test medium indicated utilisation of the plant acid. A control medium was necessary because utilisation of acidic substances in the yeast extract also led to a slight rise in pH.

d) Reaction in litmus milk.

Skimmed milk to which litmus had been added was sterilised by steaming for 1 hr. on three successive days. Cultures were examined after 1, 2 and 6 weeks.

e) Utilisation of amino acids.

A medium was devised which would demonstrate the ability of the organisms to utilise amino acids as the sole source of carbon and nitrogen. Casamino acids (not vitamin-free) (Difco), 5 g.; were added to the basal medium broth from which the yeast extract had been excluded. The development of turbidity indicated that a positive result had been obtained.

f) /

f) Nitrogen requirements.

1) Growth on mineral nitrogen.

For nutritional studies it was desirable to obtain a basal mineral medium which would support the growth of the largest number of organisms. The medium devised by Lochhead & Chase (1943) and used by the Ottawa group was compared with five different liquid media.

Medium 1. Na citrate, 1 g.; Na acetate, 1 g.; Na succinate, 1 g.; Ca gluconate, 1 g.; $NH_4H_2PO_4$, 1 g.; K_2HPO_4, 0.08 g.; KH_2PO_4, 0.02 g.; saturated $CaSO_4$, 1% (v/v); $NaMoO_4$, 0.0002 g.; $MgSO_4.7H_2O$, 0.02 g.; NaCl, 0.01 g.; $FeSO_4$, 0.005 g.; $MnSO_4$, 0.0002 g.; distilled water, 1 l.; pH 7. The medium was autoclaved momentarily at 5 lb./sq.in. and the precipitate filtered off. After tubing and sterilisation, filter-sterilised glucose was added to give a final concentration of 0.5%.

Medium 2. As Medium 1, but the glucose was added to the medium before it was sterilised.

Medium 3. As Medium 1, but KNO_3, 1 g. was added to the medium before the pH was adjusted.

Medium 4. As Medium 1, but both the KNO_3 and glucose were added before the pH adjustment.

Medium /

Medium 5. The medium of Lochhead & Chase, which contained glucose, 1 g.; K_2HPO_4, 1 g.; KNO_3, 0.5 g.; $MgSO_4$, 0.2 g.; $CaCl_2$, 0.1 g.; NaCl, 0.1 g.; $FeCl_3$, 0.01 g.; distilled water, 1 l.; pH 6.8.

Medium 6. The medium of Lochhead & Chase with the addition of $NH_4H_2PO_4$, 0.1%.

Forty-nine rhizosphere isolates were inoculated into the six media. The inoculum was one drop of a dilute suspension of sterile tap water. The tubes were examined for visible turbidity at daily intervals. Very slight turbidity which was difficult to observe was considered negative. For the purposes of the comparison, the turbidity readings were divided into two groups: fair and good.

The results in Table 2 show that more organisms developed in the complex media than in the simpler medium employed at Ottawa. The increase in total number of organisms growing in Medium 4 but not in Medium 5 is about 10%. However the increase in organisms producing good turbidity in Medium 4 is approximately 20%. It seems probable that certain organisms, which are unable to utilise glucose, require the plant acids.

Medium 4 was selected for determining the ability of organisms to grow with inorganic nitrogen sources, without the addition of any organic nitrogenous material. The other five media appeared to be less suitable.

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Table 2. Comparison of growth of 49 rhizosphere isolates in media containing only mineral nitrogen.

Medium	Fair turbidity	Good turbidity	Total showing turbidity
1	7	16	23
2	9	16	25
3	11	15	26
4	12	15	27
5	16	6	22
6	12	12	24

2) Organisms requiring amino acid nitrogen.

Medium 4 was used to which vitamin-free casamino acids (B.D.H.) had been added to give a final concentration of 0.5%.

3) Organisms requiring vitamins or growth factors.

Medium 4 was used to which 10% yeast autolysate had been added. Casamino acids (vitamin-free) were also added to give a final concentration of 0.1%. The yeast autolysate was prepared by holding 1 kg. brewer's yeast at 50° for 24 hr.; after centrifugation, the supernatant liquid was sterilised at 22 lb./sq. in.

For the studies on nitrogen requirements the organisms were incubated for 14 days. Those organisms which produced easily visible turbidity were considered positive. The assumption was proved to be correct by serial sub-culture in the same media. Less than 2% of the organisms showed different requirements during the repeated sub-cultures. The method was therefore considered suitable for the purpose of this investigation.

It has been stated earlier that the original inoculum from the stock solution was one drop of a slightly turbid suspension in sterile tap water. In order to investigate the possibility of Edinburgh tap water containing organic matter which would interfere with the results, a control series was set up in which distilled water containing 0.85% NaCl/

NaCl was used as the diluent. No differences were obtained between the two diluents, and tap water was therefore used for this purpose.

g) Nitrate reduction test.

The following medium was used since Dye (1958) had found it to be the most suitable of several which he tested. $NH_4H_2PO_4$, 0.5 g.; K_2HPO_4, 0.5 g.; $MgSO_4.7H_2O$, 0.2 g.; NaCl 5 g.; Na succinate, 2 g.; KNO_3, 1 g.; yeast extract, 1 g.; distilled water 1 l.; pH 6.8.

The production of nitrite was determined at 1, 3 and 7 days using the sulphanilic acid and α-napthylamine method (Society of American Bacteriologists 1955).

h) Denitrification.

Stab inoculations were made into 3 ml. of the following medium contained in $\frac{1}{2}$ in. tubes. KNO_3, 2.5 g.; Ca gluconate, 5 g.; Vitamin-free casamino acids 1 g.; yeast autolysate 100 ml.; agar (N.Z. Davis), 15 g.; tap water 900 ml. The cultures were examined for gas production at 1, 3, 7 and 14 days. Early examination of the cultures was necessary, the nitrogen sometimes being absorbed by the medium or diffusing into the atmosphere.

Certain other tests, which were used infrequently, are described in the appropriate sections.

B) /

B) Plant studies.

Most of the plant growth experiments were carried out in a glasshouse which had an average temperature of about 20°. No supplementary light was applied but electrical heating maintained the average temperature during spring and autumn. Certain experiments were carried out in an unshaded part of a garden and others in a shaded box beside the glasshouse.

Untreated seeds were planted directly from the packet in which they were received from the supplier. Clover seeds which were required in a sterile condition were treated by the alcohol - $HgCl_2$ method described by Allen (1951). After sterilisation the seeds were germinated on Medium 79 of Fred & Waksman (1928) at 27° for 3 days. Seedlings which did not reveal contamination were transferred to the growth medium.

The plant nutrient solution used was a modification of one (p. 189) given by Hewitt (1953). The Fe citrate used by Hewitt provided an energy source for bacteria and it was replaced by an equivalent amount of $FePO_4$. This proved to be unsuitable. It became unavailable to the plant when the pH of the nutrient solution began to rise and iron deficiency symptoms appeared. Therefore in all the experiments 16 p.p.m. Fe sequestrene was added in place of Fe citrate.

The/

The experiments were carried out under four different conditions. The plants were grown either on agar or in sand, soil or nutrient solution.

1) Experiments with agar as the supporting medium.

Preliminary experiments were conducted into the concentration of agar most suitable for surface root growth. Various concentrations of agar were added to the nutrient solution and after solidification seedlings were placed on the agar. Dilute agars permitted the penetration of the root and a normal development of organisms on the buried root did not take place. Concentrated agars appeared to bind moisture strongly and normal root growth did not occur. An agar concentration of 2% was found to be the most suitable and was used in all the agar experiments. The agar was used as slopes which were made as long as possible to provide the maximum surface for root development. The bottom half of the 1-in. tubes in which the plants were grown was sheltered from direct sunlight with brown paper.

2) Experiments in sand.

Fairly coarse sand was thoroughly washed with 2N HCl and then rinsed with tap water until the chloride ion concentration became normal. The sand was dried and then placed in 1200 ml. capacity glazed earthenware jars. The top of the jar was sealed with grease-proof paper. The

sand/

sand and container were then sterilised by autoclaving at 22 lb./sq. in. for 1 hr. on three successive days. After sowing the seed, nutrient solution was added to give a moisture content of about 70% of the water holding capacity. During the experiment the moisture content was maintained with sterile distilled water. At the time of sowing the seed the jars were inoculated with 1 ml. of 1/100 dilution of the soil being used for the experiments.

One experiment was carried out under aseptic conditions in pint milk bottles. A layer (0.5 in.) of washed gravel was placed at the bottom of each bottle to facilitate aeration and drainage. A piece of glass tubing (0.5 in.) stretched from the gravel to the cotton wool plug at the mouth of the bottle. This enabled liquids to be added directly to the nutrient solution without coming in contact with the plants. A layer of sand (2 - 3 in.) covered the gravel. Each bottle was inoculated with 0.5 ml. of a dilute aqueous suspension of the pure culture.

3). Soil experiments.

Fresh soil was passed through a 2 mm. sieve and placed in clean plant pots. The seeds were sown immediately. During the growth period soil was moistened regularly with tap water, but not allowed to become water-logged.

4) /

4) Experiments in nutrient solution.

The 1200 ml. glazed earthenware jars were filled with nutrient solution. A circular piece of ½ in. black rubber sheet, with a diameter the same as the internal diameter of the jar, was placed inside the jar. It was supported by stiff wire which overlapped the side of the jar.

The seeds which were placed in ¼ in. holes in the rubber sheeting were supported in the early stages of development by sterile nylon gauze. The gauze which was dipping into the nutrient solution kept the seed moist until the rootlet emerged. Air from a Proctor pump was blown through an aquarium dispenser which lay at the bottom of the jar. The level of the solution was maintained with sterile distilled water. Occasionally it was replenished with sterile nutrient solution. Each jar was inoculated with 1 ml. of 1/100 dilution of the experimental soil at the beginning of each experiment.

THE CHARACTERISTICS OF THE PREDOMINANT GRAM-NEGATIVE BACTERIA OCCURRING IN SOIL.

Table 3. Some properties of the predominant Gram-negative bacteria of the rhizosphere.

Genus or group	OXIDATION									Denitrification	Adequate source of nitrogen			Flagella	Litmus milk
	Glucose	Fructose	Xylose	Maltose	Sucrose	Malate	Citrate	Tartrate	Amino acids		Mineral	Amino acid	Yeast extract		
Pseudomonas	+	+	+	(+)	(+)	+	(+)	(+)	+	(-)	+	+	+	polar	P,K,r,n.
Pseudomonad A	+	+	(-)	+	(+)	+	+	(+)	+	-	-	(+)	+	"	P,n.
Pseudomonad B	-	-	-	-	-	+	(+)	(+)	+	-	(+)	(+)	+	"	R,n.
Xanthomonad	+	+	(+)	+	+	+	(+)	(+)	+	-	-	(+)	+	"	P,K,n.
Agrobacterium	+	+	+	+	+	+	+	+	+	-	+	+	+	peri-trichous	B.
Flavobacterium	+	(+)	+	+	+	+	+	+	+	-	-	(+)	+	"	K,n.
Achromobacter	+	+	(+)	+	+	+	(+)	+	+	-	-	(+)	+	"	K,n.
Alcaligenes	-	-	-	-	-	+	+	+	+	-	(+)	+	+	"	K.
Aeromonas	F	+	+	+	+	+	+	(+)	+	-	+	+	+	polar	Acid clot
Aerobacter	F	+	+	+	+	+	+	+	+	-	+	+	+	peri-trichous	Acid clot
Bacterium herbicola	F	+	+	+	+	+	+	+	+	-	+	+	+	"	Acid clot
Cytophaga	+	+	+	+	+	+	(-)	(+)	+	-	(+)	(+)	+	-	P,R,n.
Chromobacterium	+	*	*	*	*	*	*	*	+	-	+	+	+	*	P.

KEY: P = proteolysis, K = alkalinity, R = reduction, N = no action, B = browning — Capital lettering indicates predominant type.

(+) majority of strains positive
(-) " " " negative
\+ all strains positive
\- " " negative

F = fermentation (only glucose examined)

* = examination not carried out

The characteristics of the predominant Gram-negative bacteria occurring in soil.

The characteristics of the organisms which were encountered in this study and the groups into which they were classified is shown in Table 3. Occasionally a bacterial strain was isolated which did not possess the characteristics given in Table 3. For instance, some strains were non-motile; some strains possessed a green or pink pigment which was lost on repeated subculture and certain strains showed litmus milk reactions not shown in Table 3. These unclassified types never occurred as a numerically important group in the rhizosphere microflora and details of their properties have not been included. Variation in physiological characteristics, e.g. rate of acid production from sugars, amount of growth on agar slopes, were frequently encountered in these studies. These variations did not appear to be significant.

1) Pseudomonas

The properties of the organisms placed in this genus, indicated that they resembled closely Pseudomonas fluorescens Migula and related organisms. A large proportion of the strains produced in the media used a green fluorescent water-soluble pigment. The pigment was usually visible in daylight but more easily seen with the aid of the ultra-violet lamp.

Experiments/

Experiments were not carried out to determine whether those strains which did not produce the pigment on ordinary media would do so on iron-deficient media. Media with a low content of iron are known to stimulate the production of the pigment (Paton 1956). Strain variation was shown in disaccharide and organic acid oxidation and in litmus milk reaction.

For the purpose of identification the organisms were divided into types which appear on plant roots under certain conditions. The differentiation was based on litmus milk reaction:-

Pseudomonas	Type	I	Proteolysis
"	"	II	Alkalinity
"	"	III	Reduction
"	"	IV	No change

2) Pseudomonad Group A

The organisms placed in this group differ from the Pseudomonas strains in not being able to grow with an inorganic nitrogen source. They required amino acids and a few strains required growth factors. In addition no fluorescent pigments were observed. Their relationship to the xanthomonads will be discussed later.

Classification/

Classification of the different types which appeared on plant roots under certain conditions was based on the simplest nitrogen source supporting growth and the litmus milk reaction.

		Simplest nitrogen source supporting growth	Litmus milk reaction
Pseudomonad A	Type I	Amino acids	Proteolysis
" "	" II	" "	No change
" "	" III	Yeast extract	Proteolysis

3) Pseudomonad Group B

The organisms placed in this group differed from Pseudomonas strains in being unable to oxidise carbohydrates. Colonies were not fluorescent and proteolytic activity was not observed. Some strains also differed from the Pseudomonas strains in requiring for growth amino acids or growth factors. Amongst the properties by which the organisms differed from Pseudomonas stutzeri (van Neil & Allen 1952) were the colonial morphology and lack of denitrifying ability.

The following types were recognised:-

		Simplest nitrogen source supporting growth	Litmus milk reaction
Pseudomonad B	Type I	Mineral nitrogen	Reduction
" "	" II	" "	No change
" "	" III	Amino acids	"
" "	" IV	Yeast extract	"

4) /

4) Xanthomonad

According to Dye (1958) organisms in the genus Xanthomonas cannot be differentiated by laboratory tests. Species differences are based on plant pathogenicity tests alone. Dye considered that organisms not possessing the group characteristics could not be placed in the genus.

The table below indicates some differences which existed between the plant pathogenic group and the rhizosphere group. These differences warrant the exclusion of the organisms from the genus Xanthomonas.

Characteristic	Plant pathogen group (Dye)	Rhizosphere group
Growth on media containing glucose	Copious slime	No slime
Litmus milk reaction	Proteolysis	Proteolysis or Alkalinity or No change
NO_3 reduction	Negative	Positive
Yellow pigment	Permanent	Frequently lost with repeated subculture

The strains which lost their pigmentation on repeated sub-culture became indistinguishable from Pseudomonad A strains. They will however be referred to as Xanthomonads since they possessed the pigment when they were initially isolated from the rhizosphere.

		Simplest nitrogen source supporting growth	Litmus milk reaction
Xanthomonad	Type I	Amino acids	Proteolysis
"	" II	" "	No change
"	" III	Yeast extract	Proteolysis
"	" IV	" "	Alkalinity
"	" V	" "	No change

5) Agrobacterium

The properties examined of the organisms placed in this genus indicate that they closely resembled Agrobacterium radiobacter (Beijerinck) Conn.

6) Flavobacterium

Organisms placed in this genus closely resembled the type species, Flavobacterium aquatile Frankland & Frankland. They differed from the type species in being motile and in not showing reduction of litmus milk. An early definition of the genus (Bergey's Manual 1923) indicated that the ammonium ion could be used as the sole source of nitrogen. Weeks (1955) revised the description of the genus and the strains he examined were unable to grow with the ammonium ion as the sole nitrogen source.

The following types were recognised:-

		Simplest nitrogen source supporting growth	Litmus milk reaction
Flavobacterium	Type I	Amino acids	Alkalinity
"	" II	Yeast extract	"
"	" III	" "	No action

7) Achromobacter

Unpigmented organisms, capable of oxidising carbohydrates, requiring organic nitrogen for growth and possessing peritrichous flagella were placed in this genus. The arrangement of the flagella was the only feature for distinguishing the organisms in this genus from those placed in Pseudomonad Group A.

The following types were encountered on plant roots under certain conditions:-

		Simplest nitrogen source supporting growth	Litmus milk reaction
Achromobacter	Type I	Amino acids	Alkalinity
"	" II	Yeast extract	"
"	"" III	" "	No action

8) Alcaligenes

The properties of the organisms placed in this genus closely resembled the type species of the genus, Alcaligenes faecalis Castellani & Chalmers. A large proportion of the strains/

strains were capable of good growth on media which contained only inorganic nitrogen sources. This observation supported the work of Ulrich & Needham (1953) who found that Alcaligenes faecalis strains would grow well on an inorganic nitrogen medium which contained glucose, sodium acetate and sodium citrate. Denault, Cleverdon & Kulp (1953) however, reported that ammonium nitrogen only supported slight growth of Alcaligenes faecalis on a sodium lactate mineral salts medium.

The following types were recognised:

		Simplest nitrogen source supporting growth	Litmus milk reaction
Alcaligenes	Type I	Mineral nitrogen	Alkalinity
"	" II	Amino acids	"

9) Aeromonas

The few organisms placed in this genus possessed biochemical and physiological characteristics very similar to the previously described species (Miles & Miles (1951) and Bergey's Manual 1957). The organisms fermented lactose and gave a positive reaction to both the Voges-Proskauer and methyl red tests (Society of American Bacteriologists 1955). They were however unable to peptonise litmus milk and did not produce gas during the fermentation of glucose.

10) /

10) Aerobacter

The tests carried out indicated that the organisms very closely resembled Aerobacter aerogenes (Kruse) Beijerinck. No differences were observed.

11) Bacterium herbicola

These organisms were very similar to Bacterium herbicola Düggeli whose properties were reviewed by Mack (1936). Mack described her organisms as possessing two flagella. The organisms encountered in this study possessed several peritrichous flagella.

12) Cytophaga

Strains placed in this genus possessed the characteristics of the genus Cytophaga Winogradsky. Some strains showed only slight "creeping" motility on the surface of solid media. Similarly, "flexing" movements in hanging drop preparations were not always observed. The cells appeared to have a lower refractive index than eubacterial cells. No microcysts or fruiting bodies were observed.

		Simplest nitrogen source supporting growth	Litmus milk reaction
Cytophaga	Type I	Mineral nitrogen	Reduction
"	" II	" "	No change
"	"" III	" "	Proteolysis
"	" IV	Amino acids	"
"	" V	Yeast extract	Reduction
"	" VI	" "	No change

13) /

13) Chromobacterium

The examinations carried out indicated that the strains closely resembled Chromobacterium lividum Eisenberg whose characteristics were reviewed by Sneath (1956). Some unpigmented variants of Chromobacterium lividum, producing tough gelatinous colonies were isolated occasionally.

EXPERIMENTS AND RESULTS.

Experiments and Results

Experiment 1. The rhizosphere microflora of oats, grass and clover at different stages of plant development.

The review of literature indicates that few workers have investigated the rhizosphere microflora of various plant species at different stages during plant development. The object of this experiment was to obtain more data on this aspect of the subject.

The plants were grown in nutrient solution. One seed was placed in each of five holes in the rubber sheeting and each seed type was sown in three jars. The seeds used were:- Sun II oats, English broad leaved red clover and perennial ryegrass. The nutrient solution was inoculated with a medium loam soil from Boghall Farm.

The oat root samples were examined at 3, 6 and 12 weeks. The grass and clover plants developed slight symptoms of iron deficiency at 6 weeks and this examination was omitted. The use of Fe sequestrene was introduced at this stage.

After a preliminary isolation of about 500 cultures from C.V. agar, 79 cultures representing the numerically significant colonial types appearing on the plates, were selected for further study.

In addition to carrying out the tests detailed in the Materials and Methods section, these organisms were examined for/

for the utilisation of xylan and the fixation of atmospheric nitrogen. Xylan was of interest because it occurs in large quantities in plant roots. To the basal medium (p. 21) containing bromo-cresol-purple and 1.5% agar, was added 0.5% of partially purified xylan, kindly donated by Dr. Aspinall, Department of Chemistry, Edinburgh University. Plates were inoculated with the 79 organisms and incubated at 22°. In no case did a zone of clearing appear around the colony and no acid was produced, thus showing that xylan was not attacked.

As a presumptive test for nitrogen fixation the organisms were examined for growth on nitrogen-free silica gel. The gel was prepared in a similar way to that used by Smith (1951). A 10% solution of sodium metasilicate (B.D.H.) was passed through a column of Amberlite Resin 1R - 48 (OH) (B.D.H.). To the silicic acid produced was added the constituents of Medium 1 (p. 24) without the $NH_4H_2PO_4$. The pH of the medium was raised to approximately 6.8, with sterile 0.1N NaOH. The medium was allowed to solidify in Petri dishes. The 79 cultures were inoculated onto the medium and incubated at 22°. No growth was observed and it was concluded that under these conditions the organisms were unable to fix sufficient nitrogen for their growth requirements. The organisms might be capable of fixing nitrogen in a similar manner to that described by Proctor & Wilson (1958) for

strains/

Table 4. The development of bacteria on the roots of oats, grass and clover grown in nutrient solution.

	OAT				GRASS			CLOVER			
	Seed	Rhizosphere			Seed	Rhizosphere		Seed	Rhizosphere		Soil inoculum
		Age of plants (weeks)				Age of plants (weeks)			Age of plants (weeks)		
		3	6	12		3	12		3	12	
Total count S.E.Y.E. agar*	32	190	450	1200	7.2	111	330	0.016	158	210	7.4
Gram-negative count C.V. agar*	0.024	33	140	360	0.088	45	41	0.0035	41	28	0.14
% Gram-negative	0.1	17	30	29	1	41	12	22	26	13	2
					Gram-negative organisms (%)						
Pseudomonas	31	18	-	20	45	42	83	-	16	29	61
Pseudomonad A	44	-	29	-	-	-	-	-	13	-	25
Pseudomonad B	-	25	29	-	-	33	-	-	65	18	-
Xanthomonad	25	12	14	-	-	5	-	-	2	25	-
Agrobacterium	-	9	-	-	-	-	-	-	-	-	-
Achromobacter	-	-	21	9	30	11	-	-	-	14	-
Alcaligenes	-	9	-	65	-	-	-	-	-	14	-
Aeromonas	-	-	-	-	-	-	-	48	-	-	-
Aerobacter	-	-	-	-	25	-	-	-	-	-	-
Bacterium herbicola	-	-	-	-	-	-	-	52	-	-	-
Cytophaga	-	18	7	6	-	9	17	-	2	-	-
Non-viable	-	9	-	-	-	-	-	-	2	-	14

* = x 10^{-6} per { 10 seeds / complete root system.

Table 5. The nutritional classification of bacteria isolated from the rhizosphere of oats, grass and clover.

Plant	Sample	Adequate source of nitrogen		
		Mineral	Amino acid	Yeast extract
		%	%	%
OAT	Seed	31	27	42
	Rhizosphere 3 weeks	37	27	36
	" 6 "	36	43	21
	" 12 "	62	19	19
GRASS	Seed	70	30	-
	Rhizosphere 3 weeks	84	5	11
	" 12 "	100	-	-
CLOVER	Seed	100	-	-
	Rhizosphere 3 weeks	70	15	15
	" 12 "	43	57	-
	Soil inoculum	68	32	-

strains of Achromobacter.

The occurrence of the different Gram-negative groups on the seed and roots of oats, grass and clover is shown in Table 4. Details of the microflora of the soil with which the jars were inoculated is also given. Table 5 shows the nutritional classification of the organisms.

The marked increase in the proportion of Gram-negative bacteria in all of the rhizosphere samples supports the findings of other workers. The soil used as inoculum contained an unusually low percentage of Gram-negative organisms. The preferential effect on the Gram-negative types was greatest when the plants were growing actively. After 12 weeks, development of the clover and grass plants was not taking place and a reduction in the percentage of Gram-negative organisms was observed. After 12 weeks the oat plants were developing rapidly, the early-flowering stage had been reached and the proportion of Gram-negative organisms remained large.

The seed microflora did not seem to have any influence on the rhizosphere population. The oat and grass seed supported only a very small proportion of Gram-negative bacteria. Although the clover seed harboured a large proportion of Gram-negative organisms, the dominant types isolated were facultative anaerobes. This type of organism

was

was not detected in the clover rhizosphere at later stages of plant development.

The occurrence of organisms in the genus Pseudomonas in all except one of the samples examined indicated that they were normal inhabitants of the rhizosphere. Although they were the predominant Gram-negative type in the inoculum soil, they did not usually become the dominant type in the rhizosphere. Proteolytic strains were the most abundant type encountered.

Of considerable interest was the development in most rhizospheres of organisms which were apparently unable to oxidise carbohydrates. These organisms utilise plant acids or amino acids as energy sources. This suggested that one or both of these types of compounds were available to the organisms in the rhizosphere region. The frequent occurrence of this type of organism in rhizosphere samples indicated that Pseudomonad B strains are normal inhabitants of the rhizosphere of the plants examined. Their source is unknown since they were not detected in the inoculum soil or on the seed. Alcaligenes strains, which occurred less frequently than the Pseudomonad B strains did not appear to be normal rhizosphere inhabitants.

The repeated occurrence of Achromobacter and Xanthomonad strains in rhizosphere samples indicated that their growth was promoted by the plant root. They were not detected in the/

the soil used for the inoculum or on the clover and grass seed. None of the types appeared to be of outstanding importance even though large proportions of these groups were sometimes encountered.

Although Cytophaga strains were present in most of the rhizosphere samples, they never occurred in large numbers. This suggested that they were normal rhizosphere inhabitants, but were unable to compete effectively with the other groups for energy or nitrogen sources. The regular occurrence of Cytophaga strains indicated that chance contact between the root and the organism was not an important factor.

The nutritional classification indicated that the growth of organisms which required growth factors was preferentially promoted in the oat rhizosphere. This development could be attributable to two factors. 1) The release of growth factors by the root. 2) The liberation of excess growth factors by organisms capable of growth using mineral or amino acid nitrogen as the sole nitrogen source. This problem will be considered later. In spite of this nutritional trend, great variation was observed in the bacterial group composition of the oat rhizosphere samples. This indicated that the rhizosphere microflora of different oat plants were made up of different bacterial types.

Although only a slight difference from the soil inoculum was/

was observed in the nutritional classification of the clover rhizosphere at 3 weeks, the growth of strains requiring amino acid nitrogen was promoted after 12 weeks. This development indicated that amino acids had become available to the rhizosphere organisms after active development of the clover plant had ceased.

The presence of grass roots seemed to exert an influence on organisms capable of growth on mineral nitrogen media. It seemed probable that organic nitrogen sources were not available to the rhizosphere organisms. Non-nitrogenous energy sources, sugars or plant acids, were presumably enabling bacterial development to take place.

The results, which have been discussed, failed to show any trend which was common to the three types of plant examined. No correlation was obtained between the bacteria in the rhizosphere and those on the seed or in the soil with which the pots were inoculated. The genera which occurred repeatedly were considered to be normal rhizosphere inhabitants. The apparently haphazard proportions of these groups indicated however that there were rapid changes in the types of organisms developing on the root. Alternatively, there was the possibility that two plants did not harbour the same organisms about their roots, even if the nutritional classification was similar. There was no correlation between the composition of the rhizosphere microflora and the rate of plant growth.

Experiment 2. The root microflora of oats growing in soil, sand and nutrient solution.

Oat plants were grown in soil, sand and nutrient solution. The soil was the same as that used in Experiment 1. The sand and nutrient solution were inoculated with 1 ml. of 1/100 of the soil. The plants were harvested after 6 weeks and examinations made of representative Gram-negative isolates.

The object of the experiment was to show the effect which the environment of the plant roots exerted on the rhizosphere microflora. Two aspects of the problem appeared important. Firstly, the plants growing in sand and nutrient solution developed more rapidly than those in the soil. Does the more rapid plant growth affect the rhizosphere bacterial population? Secondly, does the presence of soil and the micro-organisms contained therein have an effect? In the sand and nutrient solution it was likely that the bacteria would be nourished solely by the plant roots. In soil, organic matter in the soil could play a part. The nutrient solution in which the plants were grown was also examined. If the organisms in the solution should be similar to those on the root, a simple method would be available for the examination of one particular plant during development.

The occurrence of organisms in the different groups is given in Table 6 and the nutritional classification is shown in/

Table 6. The development of bacteria on oat roots grown in soil, sand and nutrient solution.

	RHIZOSPHERE				
	Growth medium			Nutrient solution	Soil inoculum
	soil	sand	nutrient solution		
Total count S.E.Y.E. agar*	500	380	500	0.88+	7.4
Gram-negative* count C.V. agar	180	55	300	0.38+	0.14
% Gram-negative	36	14	60	43	2
	Gram-negative organisms (%)				
Pseudomonas	48	27	10	11	61
Pseudomonad A	-	-	-	-	25
Pseudomonad B	-	-	20	26	-
Xanthomonad	11	-	13	44	-
Flavobacterium	-	-	12	-	-
Achromobacter	-	54	7	11	-
Alcaligenes	6	5	-	6	-
Aeromonas	11	-	-	-	-
Aerobacter	24	-	25	-	-
Cytophaga	-	14	-	-	-
Chromobacterium	-	-	13	2	-
Non-viable	-	-	-	-	14

* = x 10^{-6} per complete root system.

+ = x 10^{-6} per ml.

Table 7. The nutritional classification of bacteria isolated from oat roots growing in soil, sand and nutrient solution.

Adequate source of nitrogen	RHIZOSPHERE			Nutrient solution	Soil inoculum
	Growth medium				
	soil	sand	nutrient solution		
	%				
Mineral	85	41	68	45	68
Amino acid	10	59	32	44	32
Yeast extract	5	-	-	11	-

in Table 7.

Although strains of Pseudomonas were present in the original soil, their repeated occurrence in the rhizosphere samples supported the observation made in Experiment 1 that they were normal inhabitants of the rhizosphere. The source of the other groups which occurred regularly was unknown. It seemed likely however that their development was associated specifically with the plant root. On the other hand, Pseudomonad A strains did not develop in the rhizosphere even though they were present in appreciable numbers in the control soil.

Facultative anaerobes were not isolated from the rhizosphere samples in Experiment 1. The results of the present experiment indicated that under certain conditions facultative anaerobes could occur in large proportions in the oat rhizosphere microflora. Their source was unknown.

The increase in the sand rhizosphere of organisms requiring amino acid nitrogen, associated with an increase in Achromobacter strains, was not observed on the roots grown in nutrient solution. Furthermore, the proportion of Gram-negative organisms in the sand rhizosphere was very low compared with the proportion on the roots of plants in the nutrient solution. It is possible that the sand environment may have affected the release of amino acids by the roots. The

The promotion of growth in the soil rhizosphere of organisms capable of using mineral nitrogen indicated that non-nitrogenous energy sources were available in the rhizosphere region.

The results obtained in this experiment indicated that the three environments promoted the growth of a different microflora. The same groups of organisms usually developed in two or all three environments, but the proportions of the groups were different. These findings support the work of Pántos (1957), who reached the same conclusions after examining the microflora of wheat plants grown in soil, sand and nutrient solution.

The microflora of the roots and the nutrient solution in which they were growing showed similarities. The nutrient solution microflora could not however be considered to be representative of the rhizosphere microflora.

Experiment 3. An examination of the microflora of parts of an oat root.

The object of this experiment was to obtain data on the microflora of different parts of the oat root system. From the data obtained, certain aspects of root physiology, involving exudates could be considered.

An oat plant was grown in the same soil as had been used in Experiments 1 and 2. The plant was harvested after 7 weeks. Care was taken not to allow any part of the root to become contaminated with soil in which it had not come in contact during growth. The root system was cut into 4 portions described as: 1) The crown region, which was a 2 in. section of root starting at the soil surface; 2) The tip region which was about 1 in. in length from the tips; 3) Mid-upper and 4) Mid-lower were the remainder of the root system divided into two sections.

Examination of the isolates from the four sections of the root was made and the results are recorded in Tables 8 and 9.

Although the growth of Gram-negative organisms was preferentially promoted in all four sections, the development was much smaller in the mid-upper region. No explanation can be offered for this smaller effect.

The occurrence of the same four groups of organisms in the crown region and mid-sections suggested that a similar rhizosphere/

Table 8. The development of bacteria on different sections of the oat root.

	SECTION				Control soil
	Crown region	Mid-upper	Mid-lower	Tip region	
Total count S.E.Y.E. agar*	33	20	22	51	740+
Gram-negative* count C.V. agar	6	1.1	3.4	10	14+
% Gram-negative	18	5.5	15	20	2
	Gram-negative organisms (%)				
Pseudomonas	10	27	57	50	61
Pseudomonad A	-	-	-	-	25
Pseudomonad B	-	-	6	-	-
Xanthomonad	30	37	17	-	-
Flavobacterium	20	18	11	-	-
Achromobacter	-	-	-	-	-
Alcaligenes	-	-	-	10	-
Cytophaga	40	18	9	40	-
Non-viable	-	-	-	-	14

* = x 10^{-6} per section of root.

+ = x 10^{-6} per g.

Table 9. The nutritional classification of bacteria isolated from sections of an oat root.

Adequate source of nitrogen	SECTION				Control soil
	Crown region	Mid-upper	Mid-lower	Tip-region	
	%				
Mineral	50	45	72	100	68
Amino acid	50	55	28	-	32
Yeast extract	-	-	-	-	-

rhizosphere effect was being promoted. The nutritional classification supported this indication. The slight nutritional change in the mid-lower section became more definite in the tip region, where all of the organisms isolated were capable of growth on mineral nitrogen media. The absence of organisms requiring organic nitrogen indicated that either these compounds were not available or the organisms capable of using mineral nitrogen had successfully competed for and assimilated the compounds. The latter seemed less likely since the organisms requiring amino acid nitrogen had developed higher up the root. The large microbial population in the root tip region showed that energy sources were available to the organisms. If amino acids were not available in the root tip region, the occurrence of Alcaligenes strains indicated that plant acids were the available energy sources. These conclusions do not agree with Timonin & Lochhead (1948) who found a larger proportion of organisms requiring amino acids in the tip region than elsewhere on the root system of a tobacco plant.

In the present experiment organisms requiring yeast extract were not encountered and strains of Flavobacterium appeared as a dominant group. Different observations were made in Experiments 1 and 2 where Flavobacterium strains

rarely/

rarely occurred and organisms requiring yeast extract were numerous. This variation emphasises that plants of the same species grown in the same soil and environment do not necessarily harbour the same rhizosphere microflora.

Experiment 4. The effect of pure cultures of bacteria on clover plants growing in nine nutritional environments.

The literature review reveals that very little is known of the effect of the plant environment on the individual types of bacteria found in the rhizosphere. Similarly, there is very little information concerning the influence which pure cultures of bacteria exert on plants.

The object of this experiment was to show the effect of nine nutritional plant environments on: 1) sterile plant growth, 2) the development of the rhizosphere bacteria and 3) the plant-bacterial relationship.

The experiments were carried out in sand culture under aseptic conditions in pint milk bottles. Three sterile clover seedlings were added to each bottle. The nutrient solutions in which the plants were grown were adaptations of modified Hewitt's nutrient solution (M.H.N.S.) referred to on page 28. Deficiency media were prepared as detailed by Hewitt. The effect of organic matter was examined by adding glucose, sodium malate or Casamino acids. These compounds were selected because similar substances are known to be liberated by plant roots under certain conditions. Aqueous solutions of the compounds were added in 4 equal amounts at intervals of 2 weeks to give the nutrient solution the final concentration given below.

Medium/

Medium	Constituents
A	M.H.N.S.
B	Potassium deficient M.H.N.S.
C	Phosphate deficient M.H.N.S.
D	Nitrogen deficient M.H.N.S.
E	M.H.N.S. + 0.2% glucose
F	M.H.N.S. + 0.02% Na malate
G	M.H.N.S. + 0.2% Casamino acids
H	M.H.N.S. + 0.02% Casamino acids
J	M.H.N.S. + 0.02% glucose

In addition to rhizosphere strains three organisms known to be capable of fixing large amounts of nitrogen under certain conditions were also included.

The following 12 cultures were each inoculated into bottles containing the different nutrient media. Uninoculated controls were also prepared.

Genus	Type or strain	Source
Pseudomonas	II	Rhizosphere of grass
Pseudomonas	I	" " "
Pseudomonas	IIa	" " oats
Achromobacter	II	" " "
Flavobacterium	III	" " "
Cytophaga	III	" " "
Pseudomonad B	I	" " "
Alcaligenes	I	" " "
Xanthomonad	II	" " "
Aerobacter aerogenes	M5a	University of Wisconsin
Aerobacter aerogenes	B	" " "
Azotobacter indicum	Unknown	" " "

Visual/

Visual examinations of plant growth were made at intervals of 2 weeks, up to 10 weeks. After 10 weeks the combined root systems of the 3 plants in each bottle were macerated in 1 in. tube containing 10 ml. of sterile tap water. An estimate of the development of the organisms on the roots was made by plating 1 ml. of five dilutions in Topping's medium. The dilutions were prepared by transferring one wire loopful to 10 ml. of sterile tap water. The development was assessed according to the dilution which showed a countable (30 - 300) number of colonies on the plate. This method also enabled contaminating organisms to be detected.

A comparison of the development ofthe bacteria is shown in Table 11. The effect of the different environments and of the pure cultures of bacteria on the growth of the clover plants after 10 weeks is shown in Table 10.

The following general conclusions were drawn from these experiments:

1) <u>The effect of mineral deficiencies</u>.

The severity of the mineral deficiency symptoms usually did not increase when pure cultures of bacteria were present in the root environment. More severe phosphate deficiency symptoms were induced by 3 of the strains used. These were the only observations made in the deficiency experiments in which the organisms exerted a detrimental effect on the plant./

Table 10. The effect of pure cultures of bacteria on clover plants grown in nine nutritional environments.

Inoculum	MEDIUM								
	A	B	C	D	E	F	G	H	J
	Growth of plants								
Uninoculated	++++	+++	++	++	++	+	o	+++	++++
Pseudomonas II	++++	++++	+	++	+++	++++	++++	++++	++++
Pseudomonas I	++++	++++	+	++	++++	++++	++++	++	+++
Pseudomonas IIa	++++	++++	++	++	++++	++++c	++	+++	++++
Achromobacter II	+++	++++	++	++	++++	++++	++++	+++c	++++
Flavobacterium III	+++	++++	+	++	+++	+++	++++	+++	++++
Cytophaga III	++++	++++	++	++	++++	+	+++	+++	++++
Pseudomonad B I	++++	+++	++	++	+++	++++	o	++++	++++
Alcaligenes I	++++	++++	++	++	++++	++++	++++	++++	+++
Xanthomonad II	++++	++++	++	++	++++	++++	o	++++	+++
A. aerogenes M5a	++++	++++	++	+++	++++	++++	+++	++++	++++
A. aerogenes B	++++	++++	++	+++	++++	+++	+	++++	++++
A. indicum	++++	++++	++	+++	++++	++++	+++	++++	++++

++++ = Excellent
+++ = Good
++ = Fair
+ = Poor
o = No growth
c = Mould contamination

Table 11. Comparison of the growth of pure cultures of bacteria on clover plants grown in nine different nutrient solutions.

Inoculum	MEDIUM								
	A	B	C	D	E	F	G	H	J
Uninoculated	o	o	o	o	o	o	o	o	o
Pseudomonas II	+++	++	++	++	++++	++++	++++	++++	++
Pseudomonas I	+++	+++	++++	+++	+++++	++++	+	+++	++
Pseudomonas IIa	++++	++++	+++	++	++++	+c	++++	+	+++
Achromobacter II	+++	o	+++	+++	+++	+++	+	+c	++++
Flavobacterium III	+++	+++	+++	o	++	+++	+++	++	++
Cytophaga III	+	o	+++	o	++	o	+	+	++++
Pseudomonad B I	+	o	+++	+++	+++	+++	o	o	+++
Alcaligenes I	+++	++	+++	++	+++	++	+++	+++	+++
Xanthomonad II	++	++	++	++	++++	++	o	++	+
A. aerogenes M5a	+++	+++	+++	+++++	+++++	++	+++++	+++++	+++++
A. aerogenes B	+++	+++++	+++++	++++	++++	+++++	+++++	++++	+++++
A. indicum	+	+	+	+++++	+++	++	+	+++	+++

+++++ = Very many
++++ = Many
+++ = Fair
++ = Few
+ = Very few
o = No organisms
c = Mould contamination

plant. Although the mineral deficiency conditions encouraged the development of some organisms in the rhizosphere, other types were unable to develop. In the nitrogen-deficient environment, organisms developed which were known to be capable of fixing atmospheric nitrogen under certain conditions. This multiplication resulted in a slight increase in plant growth. Steinberg (1951b) reviewed the effect of mineral deficiencies on the amino acid/protein and reducing sugar/total carbohydrate ratios in plants. Alterations in these ratios which may have taken place in these experiments did not usually seem to affect the development of the organisms. The exception was in the phosphate-deficient environment. According to Steinberg, this condition usually increases the amino acid/protein ratio in the plant. An increase in the excretion of amino acids may have taken place as a result of this condition. This could have been the reason for the fair development in the phosphate-deficient environment of certain organisms which showed less development with other deficiency conditions. Most of the bacterial strains appeared to prevent the development of slight deficiency symptoms induced in uninoculated plants by the potassium-deficient medium.

2) /

2) The effect of a concentration of glucose (E), sodium malate (F) or Casamino acids (G) which was toxic to the plant.

The glucose was assimilated by all the bacterial strains tested. Most strains of bacteria showed greater development than in the control nutrient environment without glucose. This indicated that energy sources were the factors limiting their growth. The increased multiplication of the Pseudomonad B and Alcaligenes strains indicated that either plant enzymes were carrying out the initial stages of glucose oxidation or rhizosphere conditions were conducive to glucose oxidation by these organisms, even though in vitro experiments proved negative. The sodium malate and Casamino acids were assimilated by some strains of bacteria permitting better plant development. Certain strains of bacteria appeared unable to tolerate the concentrations used. The plant did not benefit from inoculation with these organisms. There was no improvement in plant growth, compared with the control environment. Morphological changes of aerial parts were not observed in any of the plants.

3) The effect of a concentration of glucose (J) and Casamino acids (H) not toxic to the plant.

The organic additions did not bring about an improvement in the growth of sterile plants. A slight reduction of plant growth in the control environment (A), which was observed/

observed when the Achromobacter and Flavobacterium strains were inoculated, was not demonstrated when the organic compounds were present. The use by the plant of by-products of the microbial breakdown of the compounds may be the reason for the improved growth. These results confirm the work of Swaby (1942) who concluded that in complete mineral solution, sterile organic compounds do not promote plant growth. Swaby also found that the organic products of microbial action could promote plant growth, depending on their concentration.

4) Bacterial development.

The development of pure cultures of bacteria did not seem to be correlated with any known biochemical or physiological characteristics of the organisms. The lack of correlation between development and the nutritional classification indicated that nitrogen sources were not factors limiting bacterial growth.

Experiment 5. An examination of the development of pure and known mixed cultures of bacteria on clover seedlings.

The review of the literature showed that few attempts have been made to examine the development on roots of pure cultures of bacteria. In this experiment the development of organisms with known characteristics was compared in both pure and mixed culture, in an attempt to show a relationship between the biochemical and physiological characteristics of the organisms and their development in the rhizosphere.

Sterile clover seedlings growing on M.H.N.S. agar slopes were inoculated with 0.1 ml. of a slightly turbid aqueous bacterial suspension. The suspension contained either a pure culture or a simple mixed culture containing two known organisms. The number of organisms inoculated onto the seedling was determined by plating in Topping's medium.

The organisms used for the experiments possessed colony characteristics which could be used for identification purposes. Thirteen organisms were inoculated alone and in combination with each of the other 12 organisms, except those which possessed the same colony type.

The following rhizosphere organisms were used:

Genus/

Genus or Group	Type	Reference	Source	Colony type
Pseudomonas	II	Ps 2	Grass	Medium size, slight fawn, fluorescent
Pseudomonas	I	Ps 1	"	Medium size, slight fawn, fluorescent
Xanthomonad	II	Th 1	"	Medium size, pale yellow
Achromobacter	III	Ac 2	Oat	Large, pale grey, slightly mucoid
Achromobacter	III	Ac 3	"	Large, pale grey, slightly mucoid
Achromobacter	II	Ac 1	Grass	Large, pale grey, slightly mucoid
Achromobacter	III	Ac 4	Oat	Small, pale grey, irregular edge
Pseudomonad B	IV	Mo 1	"	Small, pale grey, irregular edge
Flavobacterium	II	Fl 1	"	Medium size, medium yellow
Cytophaga	II	Cy 1	"	Thin spreading yellow
Cytophaga	I	Cy 2	Grass	Thin spreading yellow
Pseudomonad B	IV	Mo 2	Clover	Small pale grey
Alcaligenes	I	Ka 1	Grass	Small, white

After 5 weeks the clover seedlings were macerated and the numbers of organisms of the 2 types which had developed on the root were determined by plate counts on Topping's medium. The error in counting the organisms on the root was undoubtedly large. Many would remain on the agar after the root was removed. Calculation of the respective multiplications of the two cultures were then made. Comparison of the development of the organisms is shown in Table 12. In the table the/

Table 12. The relative development of two cultures of bacteria inoculated simultaneously onto the same clover seedling.

Group	Type	Reference	Organisms with which the development was compared												
			Ps2	Ps1	Th1	Ac2	Ac3	Ac1	Ac4	Mo1	Fl1	Cy1	Cy2	Mo2	Ka1
Pseudomonas	II	Ps2		A	+++	+++	+	+++	+++	+++	+++	+++	++	+++	+++
Pseudomonas	I	Ps1	A		+	++	+	+	++	−	−	−	++	+++	+
Xanthomonad	II	Th1	—	−		−	−	−	−	+	++	+	+	++	++
Achromobacter	III	Ac2	—	—	+		A	A	−	++	+	+	+++	−	+
Achromobacter	III	Ac3	−	−	+	A		A	+	+	++	−	+	ND	+
Achromobacter	II	Ac1	—	−	+	A	A		o	−	+	+	+	+	+
Achromobacter	II	Ac4	—	—	+	+	−	o		A	+	+	−	ND	++
Pseudomonad B	IV	Mo1	—	+	−	—	−	+	A		−	++	+	++	+
Flavobacterium	II	Fl1	—	+	—	−	—	−	−	+		+	−	+	++
Cytophaga	II	Cy1	—	+	−	−	+	−	−	—	−		A	+	+
Cytophaga	I	Cy2	—	—	−	—	−	−	+	−	+	A		++	+
Pseudomonad B	IV	Mo2	—	—	—	+	ND	−	ND	—	−	−	—		++
Alcaligenes	I	Ka1	—	−	—	−	−	−	—	−	—	−	−	—	

KEY: +++ = development >1000X greater than companion organism.
++ = " >100X " " " "
+ = " >10X " " " "
o = " approximately same as " "
− = " >10X less than " "
— = " >100X " " " "
— = " >1000X " " " "

A = examination not carried out.
ND = no development of organisms.

the highly competitive organisms are placed in the upper left hand corner. The weakly competitive strains occur towards the lower left hand part of the table.

The Pseudomonas strains were the most competitive organisms which were tested. The Pseudomonas Type II strain competed very effectively with all the other organisms. The Pseudomonas Type I was less effective. The only known difference between these two Pseudomonas strains was the proteolytic activity of Type I which was not possessed by Type II.

The Xanthomonad strain showed a clear division in the organisms with which it was able to compete effectively. Of particular interest was the ability of the Xanthomonad to multiply more than the Cytophaga and Alcaligenes strains, each of which was capable of growth on mineral nitrogen. It was concluded that the Xanthomonad strain was effective in competing with these organisms for energy sources.

The Achromobacter strains tested were of interest in that all four strains showed broadly the same activity. They were unable to compete with the Pseudomonas strains, but except for isolated instances which were unexplainable, they competed effectively with the other organisms. The Flavobacterium strain showed similar activity.

The/

The Cytophaga strains did not compete with most strains with which they were tested, in spite of being able to utilise mineral nitrogen.

The two Pseudomonad B strains showed distinct differences in activity although no differences in characteristics of the organisms are known.

The Alcaligenes strain was unable to compete with any other strains.

On no occasion did the plating of mixed cultures on Topping's medium indicate that there were antagonistic effects between two organisms. However, Hely, Bergersen & Brockwell (1954) showed that the complete rhizosphere microflora can be antagonistic towards rhizobia introduced as a leguminous plant inoculum. The introduced rhizobia failed to establish themselves in the rhizosphere.

None of the known characteristics of the organisms appeared to account for the preferential development of some types more than others. Colony development by Pseudomonas strains on agar media was usually more rapid than by Achromobacter which was more rapid than Pseudomonad B and Alcaligenes strains. This indicated that rate of growth could be a factor involved in determining the development of some organisms more than others in the rhizosphere. This reasoning however fails to account for the/

the presence in certain rhizosphere samples of large proportions of Alcaligenes and Pseudomonad B strains.

A comparison of the bacterial count obtained after pure cultures had developed on roots, with their development after being mixed with another of the organisms used in the experiment, showed an interesting trend. Greater development of organisms which have complex nutritive requirements took place when they were mixed with others requiring only mineral nitrogen. This suggests that the second type of organism released the amino acids or growth factors required by the first. This may have been the reason for an increase, which was observed in the numbers of Achromobacter, Pseudomonad B and Xanthomonad strains. For such organisms, nitrogen compounds were perhaps the limiting factors for growth. This interpretation receives support from the results of Lochhead & Thexton (1947), Gyllenberg (1956) and Payne, Rouatt & Lochhead (1957) who found that cell-free filtrates of the organisms requiring only inorganic nitrogen supported the growth of others which required organic nitrogen.

A reduction in the development of organisms requiring only mineral nitrogen was observed when they were mixed with others requiring organic nitrogen. This suggests that the utilisation of part of energy sources by the second type of organism/

organism was limiting the development of the first. This may have been the reason for a reduction, which was observed in the numbers of Pseudomonas, Cytophaga and Alcaligenes strains which developed.

No comparison was made in this experiment between the development of pure cultures on different types of plants. The development on clover roots of isolates from different plant species did not however indicate any plant specificity.

Experiment 6. The microscopical examination of pure cultures of bacteria developing on plant roots.

This experiment was carried out with the object of comparing the development on clover roots of pure cultures of bacteria isolated from rhizosphere samples. The results obtained were examined with a view to correlating the development with (a) the results obtained in Experiments 4 and 5, (b) the nutritional classification of the organisms and (c) the occurrence of the organisms in the rhizosphere.

The experimental work was divided into two sections:-

1) Preliminary investigation using x 450 magnification.
2) Detailed investigation using x 1000 magnification.

1) Preliminary investigation.

Sterile clover seedlings were placed on M.H.N.S. agar slants and inoculated in triplicate with 0.1 ml. of a dilute suspension of the culture. The 79 cultures which were used represented all the types which occurred frequently in rhizosphere samples. After 5 - 6 weeks incubation in the glass-house the seedlings were examined for bacterial development. The use of the x 45 objective enabled examination of the roots to be made without their removal from the agar. Distortion of the light passing through the glass tube and the agar prevented a clear image/

image being obtained with phase contrast equipment. Examination with the ordinary microscope showed the development of 18 organisms on the roots. A few bacteria could be seen on the agar, close to and along the entire length of the root in the region of contact of the root with the agar. The greatest development was immediately behind the root tip in the region of root hair proliferation. Colony development appeared to take place around the root hairs.

Variation was observed in the bacterial development on the three seedlings inoculated with the same culture. Some seedlings showed poor development, distorted growth or lack of root hairs. However some variation was obtained which could not be accounted for by the appearance of the seedling.

Certain strains of the following groups showed development:-

1) Pseudomonas I
2) Pseudomonas III
3) Pseudomonas IV
4) Xanthomonad II
5) Xanthomonad IV
6) Pseudomonad A II
7) Agrobacterium
8) Achromobacter II
9) Achromobacter III
10) Aeromonas
11) Bacterium herbicola.

Certain other strains of the chief groups detailed above and all the strains examined of the remaining types did not show development.

These results indicated that strain variation existed within/

within each group. A similar observation was made in Experiment 5. Although no strains which were unable to oxidise carbohydrates developed, many strains which could utilise these substances did not develop. It appeared therefore that the supply of carbohydrates was not involved. The strains which did develop possessed all three types of nitrogen requirement. This indicated that a failure to multiply could not be associated with the nitrogen supply. These results did however show a relationship to those obtained in Experiment 5, where Pseudomonas, Achromobacter and Xanthomonad strains developed to a greater extent than the other strains tested.

2) Detailed investigation.

The use of x 450 magnification made the examination of thin films of organisms on roots impossible. An examination of the root surface at x 1000 magnification was therefore carried out.

Clover seedlings were grown on M.H.N.S. agar in Petri dishes. Each seedling was inoculated with 0.1 ml. of a dilute aqueous suspension of the organism, and the dishes were kept in a glasshouse for 4 - 5 weeks before the examination. The lids of the Petri dishes were then removed and Aniline blue stain (Jones & Mollison 1948) was pipetted/

pipetted carefully onto the root region. After 2 - 3 min. excess stain was pipetted off. The preparation was air-dried before applying the immersion oil, which was placed directly onto the root without a coverglass. This technique allowed direct observation of the root with the oil immersion objective without disturbing the seedling. The aniline blue appeared to be satisfactory for the purpose. Micro-organisms showed up well in a clear background. The plant cell walls were stained, together with certain plant cell inclusions. These inclusions were of bacterial dimensions (2 - 5μ) but their presence in sterile root cells indicated that they were not microbial in nature.

Basic fuchsin was tried in place of the aniline blue. The plant cell and agar constituents stained more readily with this substance than with aniline blue and bacterial cells on the surface of the root became less easily observed. The use of nigrosin and indian ink was also explored. These materials were readily obsorbed by the agar and the light passing through the agar was of low intensity. Organisms were difficult to observe on root surfaces.

An impression preparation was also made. A coverglass was carefully pressed onto the root growing on agar in a Petri dish. The preparation was fixed by gentle heating and stained with aniline blue. A disorganised mass of bacterial/

Table 13. Microscopical examinations of the development of pure cultures of bacteria on clover roots.

Group	Type	Number of strains examined	DEVELOPMENT				
			Very large	Large	Fair	Small	Nil
Pseudomonas	I	6	1	4		1	
Pseudomonas	II	2			1	1	
Pseudomonas	III	1	1				
Pseudomonas	IV	2		1		1	
Pseudomonad A	I	2	1				1
Pseudomonad A	II	3	1	1			1
Pseudomonad B	II	2				1	1
Pseudomonad B	IV	1				1	
Xanthomonad	II	1		1			
Xanthomonad	III	1					1
Xanthomonad	IV	1	1				
Agrobacterium		1		1			
Flavobacterium	II	1					1
Achromobacter	I	3					3
Achromobacter	II	2		2			
Achromobacter	III	2		1		1	
Alcaligenes	I	2				1	1
Cytophaga	I	1			1		
Cytophaga	II	2		1			1
Cytophaga	III	1					1
Cytophaga	IV	1					1
Aerobacter		1					1
Bacterium herbicola		1			1		

bacterial cells could be observed in the impression area. This provided little useful information and the method was discarded. Attempts to persuade a root to grow over a coverglass were unsuccessful. The dry surface of the glass prevented root development.

The development of 40 bacterial strains was examined by the aniline blue method. Four seedlings inoculated with a pure culture were grown in each Petri dish. The results recorded in Table 13 were obtained by assessing development on the four seedlings.

The results, in general, confirmed the findings of the preliminary investigation. Consideration of nutritional aspects did not elucidate the reasons for the development of some strains more than others. Variation within a given type, which could not be accounted for by seedling variation made the issue more complicated. The results however did follow a similar pattern to those obtained in Experiment 5.

The technique employed enabled a more detailed examination to be made into the specific areas of the root where bacterial multiplication took place. Multiplication was always associated with root hairs and occasionally, but to a lesser extent, with the root tip. Organisms which showed slight development produced a thin patchy film over certain root hairs. Only occasionally was a colony observed with/

with these strains. This was usually associated with a distorted root hair which may have been damaged. Intracellular materials may have thus been available to the organisms.

The strains which showed much development formed large colonies which frequently surrounded the root hairs. No development however could be shown on root hairs which were not in contact with the agar. It appeared that the agar moisture was required for bacterial growth. Development was rarely observed on parts of the stem where root hairs were not present. Organisms were frequently seen in the region of the root tip but colonies were not observed. It appeared that large amounts of materials stimulating the multiplication of bacteria were not released in the root tip region. A colonisation of sloughed-off root tip cells was not detected when these organisms were present.

These results confirm the findings of Linford (1942) who found that bacterial colonies developed mainly in the root elongation zone. They also support the work of Starkey (1938) who found that bacterial development took place on vigorous root hairs. Both of these workers were examining plant roots growing in soil and associated with the total soil flora.

Experiment 7. An examination of the rhizosphere microflora of oat plants growing in three soils in five environments.

The results obtained in Experiment 1 did not indicate that the growth of specific types of bacteria was invariably promoted in the rhizosphere of oats, grass and clover growing in nutrient solution inoculated with a Boghall Farm soil. It was suggested that chance contact between the root and organisms already present in the soil, was an important factor in rhizosphere development. The object of the present experiment was to separate the rhizosphere effect exerted by the plant from the effect of chance contact of soil organisms by the root. Differences in the rhizosphere microflora which could be correlated with plant environment or original soil microflora would enable a more accurate assessment to be made of the rôle of these factors in the development of the rhizosphere microflora.

Oat plants were grown in 3 soils in 5 environments. Rhizosphere samples were examined after 7 weeks (W sample) and 10 weeks (X sample).

The following soils were used:-

Soil 11 A sandy woodland soil obtained from the Old Wood plot, Boghall Farm. pH = 5.0 - 5.5. The soil had received no fertiliser treatment. No crops had been grown on the plot.

Soil 12 /

Soil 12 A medium-loam from Boghall Farm. pH = 6.5 - 7.0. The soil had received no special treatment and was being used in the normal rotational cropping system of the farm.

Soil 13 A medium-loam from the walled garden at Dryden. pH = 8.0. No special treatment. Used for horticultural purposes.

After sieving, each soil was added to eight 6-in. pots. The pots were then treated as follows:-

1. Normal treatment. Two pots were placed in an open unshaded area of a walled garden. This environment provided normal plant growing conditions.

2. Fertiliser treatment. The soil in two pots was intimately mixed with fertiliser which consisted of ½ oz. superphosphate; ½ oz. hoof and horn meal; ¼ oz. muriate of potash and ¼ oz. chalk. These pots then received the normal treatment.

3. Shade treatment. Two pots were placed inside a wooden slatted box, which was situated in a shaded part of the walled garden. On a fairly bright cloudy day the light intensity inside the box was approximately 125 ft. candles and the intensity outside was approximately 375 ft. candles.

4. Temperature treatment. Two pots were placed inside the glasshouse. The temperature inside the glasshouse was normally 6° - 10° higher than the outside shade temperature.

In/

Table 14. The effect of plant environment on the development of bacteria in the oat rhizosphere using Soil 11.

	TREATMENT										Control soil
	Normal		Fertiliser		Shade		Nutrient solution		Temperature		
	W	X	W	X	W	X	W	X	W	X	
Total count* S.E.Y.E. agar	15	48	152	290	48	28	240	2500	31	35	6.3+
Gram-negative* count C.V. agar	2.3	4.6	57	72	8.5	4.4	41	300	13.9	7.3	1.9+
% Gram-negative	15	10	43	25	18	16	17	12	44	20	30
	Gram-negative organisms (%)										
Pseudomonas	84	66	43	31	98	100	-	7	75	66	79
Pseudomonad B	-	-	-	-	-	-	29	57	-	-	6
Xanthomonad	-	-	17	10	-	-	15	-	3	10	6
Agrobacterium	-	-	5	-	-	-	-	-	-	-	-
Flavobacterium	-	-	-	-	-	-	7	8	-	-	-
Achromobacter	-	24	18	41	-	-	29	20	19	24	9
Cytophaga	-	10	17	18	-	-	20	8	-	-	-
Bacterium herbicola	-	-	-	-	2	-	-	-	-	-	-
Chromobacterium	16ϕ	-	-	-	-	-	-	-	3ϕ	-	-

* = x 10^{-6} per complete root system.
\+ = x 10^{-6} per g.
ϕ = gelatinous unpigmented strains.
W = rhizosphere sample after 7 weeks.
X = " " " 10 "

Table 15. The effect of plant environment on the development of bacteria in the oat rhizosphere using Soil 12.

	TREATMENT										Control soil
	Normal		Fertiliser		Shade		Nutrient solution		Temperature		
	W	X	W	X	W	X	W	X	W	X	
Total count* S.E.Y.E. agar	59	96	5200	270	33	27	470	300	750	390	58+
Gram-negative* count C.V. agar	4.8	6.4	760	123	3.3	3.7	70	36	27	33	2.8+
% Gram-negative	8	7	15	46	12	11	19	12	4	7	5
	Gram-negative organisms (%)										
Pseudomonas	15	28	-	14	36	64	8	25	30	31	44
Pseudomonad A	30	-	-	-	-	-	-	-	-	-	-
Pseudomonad B	35	30	12	10	15	-	45	44	9	-	18
Xanthomonad	10	21	24	70	10	8	25	-	15	-	10
Agrobacterium	-	-	-	5	-	-	-	-	-	-	-
Achromobacter	-	21	24	-	3	22	19	31	46	69	28
Cytophaga	-	-	-	1	36	6	3	-	-	-	-
Chromobacterium	10ø	-	40ø	-	-	-	-	-	-	-	-

* = $\times 10^{-6}$ per complete root system.

\+ = $\times 10^{-6}$ per g.

ø = gelatinous unpigmented strains.

W = rhizosphere sample after 7 weeks.

X = " " " 10 "

Table 16. The effect of plant environment on the development of bacteria in the oat rhizosphere using Soil 13.

	TREATMENT										Control soil
	Normal		Fertiliser		Shade		Nutrient solution		Temperature		
	W	X	W	X	W	X	W	X	W	X	
Total count* S.E.Y.E. agar	48	46	5200	240	9	31	136	1500	29	56	23+
Gram-negative* count C.V. agar	8.5	6	600	150	2.4	3.7	80	300	5.9	20	3.1+
% Gram-negative	12	13	12	63	27	12	59	20	20	37	14
	Gram-negative organisms (%)										
Pseudomonas	25	34	71	16	56	38	11	25	36	60	49
Pseudomonad A	-	49	-	-	-	-	-	-	-	-	-
Pseudomonad B	33	-	12	-	-	-	28	9	30	10	15
Xanthomonad	9	17	7	38	15	24	34	9	8	10	12
Agrobacterium	33	-	-	-	-	-	-	-	-	-	-
Flavobacterium	-	-	-	-	-	-	8	-	-	-	-
Achromobacter	-	-	10	27	29	22	11	50	26	20	24
Alcaligenes	-	-	-	19	-	-	-	-	-	-	-
Cytophaga	-	-	-	-	-	16	8	7	-	-	-

* = x 10^{-6} per complete root system.
\+ = x 10^{-6} per g.
W = rhizosphere sample after 7 weeks.
X = " " " 10 "

Table 17. The nutritional classification of the bacteria isolated from the oat rhizosphere.

	Adequate source of nitrogen	TREATMENT										Control soil
		Normal		Fertiliser		Shade		Nutrient solution		Temperature		
		W	X	W	X	W	X	W	X	W	X	
		%										
SOIL 11	Mineral	100	76	65	49	100	100	7	7	78	66	85
	Amino acid	0	11	18	41	0	0	41	45	20	24	15
	Yeast extract	0	13	17	10	0	0	52	48	2	10	0
SOIL 12	Mineral	58	58	40	20	87	70	24	30	30	31	54
	Amino acid	5	21	0	70	0	0	66	47	21	28	33
	Yeast extract	37	21	60	10	13	30	10	23	49	41	13
SOIL 13	Mineral	64	34	71	25	56	54	42	41	52	70	64
	Amino acid	0	66	24	59	8	14	10	53	8	30	30
	Yeast extract	36	0	5	16	36	32	48	6	40	0	6

W = rhizosphere sample after 7 weeks.
X = " " " 10 "

In addition to the above environmental variations, seedlings were also grown in nutrient solution inside the glasshouse. The nutrient solution was inoculated with 1 ml. of 1/100 dilution of the control soil.

Five oat seeds were sown in each pot. The soil was moistened with tap water as required.

The results obtained from these experiments are shown in Tables 14, 15, 16 and 17.

Experiments using Soil 11.

Although this soil possessed a lower total count than Soil 12 or 13, the counts of Gram-negative organisms in the 3 soils were similar. The resulting higher proportion of Gram-negative organisms in the soil was associated with an unexpected result. The percentage of Gram-negative organisms in the rhizosphere of plants growing in 3 of the 5 environments was lower than in the original soil. The reduction in the proportion of Gram-negative bacteria was contrary to previous findings both in this study and by other workers. Of the 3 environments promoting this effect, the normal and shade conditions produced poor plant development. The flora consisted mainly of strains of Pseudomonas and the nutritional classification showed that mineral nitrogen supported the growth of a large proportion of the organisms. It seems probable that shade conditions lowered the carbohydrate content/

content of the root. Eaton & Rigler (1946) investigated the influence of carbohydrate levels and the root surface microflora on Phymatotrichum root rot in cotton and maize. They made counts of the total bacteria, blue-green fluorescent Pseudomonas-Phytomonas types and spores of Bacillus species. The count of the fluorescent group increased and the total count decreased with increasing carbohydrate content. Harley & Waid (1956) showed that high light intensity promoted good mycorrhizal formation on the root of beech whilst poor light produced loss of resistance to parasitic infections. The rhizosphere microflora of the plants growing in nutrient solution was composed of a wider variety of types than the microflora promoted by the normal and shade conditions. The bacterial development was very large even taking into account an observed increase in the area of root surface which was associated with improved plant growth.

The improved plant growth obtained with the fertiliser and temperature treatments, also promoted the growth of a large rhizosphere microflora composed of many types of organisms. With these 2 environments there was however an increase in the proportion of Gram-negative organisms.

The occurrence of Pseudomonas strains in large proportions in most of the rhizosphere samples as well as in the/

the control soil indicated that the rhizosphere microflora was related to the dominant control soil microflora. The occurrence of the other main rhizosphere types in the control soil supports this indication. The development of certain types on the roots in the nutrient solution, which did not appear elsewhere indicated that the environment was affecting the microflora or the massive microbial development affected the percentage composition of the groups in the microflora.

The initial pH of the soil was unlikely to have any selective influence on the rhizosphere microflora. Thom & Humfeld (1932) reported that plants growing in soils with a wide pH range, tended to maintain the rhizosphere region at pH 6.5 - 7.

The differences in the rhizosphere population due to plant development were more easily demonstrated in this poor sandy soil than in the other soils, which were used. A similar observation was made by Peterson (1958) during an examination of the fungal microflora of the rhizosphere.

Experiments using Soil 12

Soil 12, which was more fertile than Soil 11 possessed a higher total bacterial count and a lower proportion of Gram-negative organisms. The growth of plants was better in the nutrient solution and with the temperature and fertiliser/

fertiliser treatments than with normal and shade conditions. The improved plant development promoted the growth of large rhizosphere populations. The only similarity which these large populations possessed was a higher proportion of organisms requiring organic nitrogen sources. The plants grown in normal and shade conditions promoted the growth of organisms which would grow on mineral nitrogen media. This effect was similar to that obtained in Soil 11 and appeared to be associated with poor plant growth.

The proportion of Gram-negative organisms was not correlated with any other observation. With the shade conditions the plant growth was poor, but a larger proportion of Gram-negative organisms developed that in either the normal or temperature treatments.

The 4 main groups of organisms in the control soil repeatedly occurred in the rhizosphere samples. This indicated that the dominant microflora of the control soil developed in the rhizosphere.

Experiments using Soil 13

In the composition of the Gram-negative microflora, of Soil 13 was very similar to Soil 12. The higher proportions of Gram-negative organisms which occurred in the rhizosphere samples of Soil 13 compared with Soil 12 were correlated with/

with a higher percentage in the control soil. Differences occurred between the rhizosphere microflora which developed in the 2 soils. In Soil 13, plants growing with temperature treatment did not promote the growth of a large total microflora. This was the same effect that was found with Soil 11. The nutrient solution and fertiliser treatment promoted better plant growth and the large bacterial populations which were obtained with the other 2 soils.

The variation obtained between the W and X samples in this soil prevented detailed conclusions being drawn concerning the composition of the rhizosphere microflora. Comparison of the W and X samples of the normal, shade and temperature treatments indicated that large qualitative but not quantitative changes had taken place. The nutritional classification was not the same as that obtained with Soils 11 and 12. The definite trend towards mineral nitrogen when plants had been growing poorly and organic nitrogen when active plant growth had taken place was not observed. The larger proportion of organisms requiring amino acid nitrogen in the X samples may have been associated with the stage of plant growth. No difference in the rate of plant growth between the 2 sampling periods was observed. This observation was not made with Soils 11 and 12.

The/

The development in most rhizospheres of the 4 main groups of organisms found in the control soil followed the trend observed with Soils 11 and 12. The development of other groups on isolated occasions was attributed to chance contamination of the normal rhizosphere microflora.

Experiment 8. An examination of the rhizosphere microflora of six field-grown plants.

The results obtained in Experiment 7 indicated that the predominant original soil microflora determined, in many cases, the composition of the rhizosphere microflora. The object of the present experiment was to support those findings by examining the rhizosphere organisms of arable crops grown in the field.

During March 1958, the microflora of 6 soils from different fields at Boghall Farm were analysed. The exact location from which the soils were taken was noted. At the beginning of August 1958 rhizosphere samples were analysed from the crop which was growing in the field.

Data on the soils and crops are given below:

Soil Number	Boghall Field reference	Type of soil	Crop
7	6	Stony, sandy	Wheat
8	4	Medium-loam	Oats
9	7	Medium-loam	Potatoes
10	South of farm on left of road	Sandy	Oats
11	Howgate Toll	Medium-loam	Potatoes
12	Woodside Cottage	Medium-loam	Oats and peas

The results obtained in the experiment are recorded in Tables 18 and 19.

Of/

Table 18. The development of bacteria in the rhizosphere of 6 field crops.

	SAMPLE											
	Soil 7		Soil 8		Soil 9		Soil 10		Soil 11		Soil 12	
	C	R	C	R	C	R	C	R	C	R	C	R
Total count S.E.Y.E. agar	10.5	440	107	140	32	17	13.4	43	6.2	19	61	210
Gram-negative count C.V. agar	3.1	84	4.4	46	3.1	2.6	3	5.5	2.0	2.3	2.7	45
% Gram-negative	30	19	4	26	10	15	22	13	32	12	4	21
	Gram-negative organisms (%)											
Pseudomonas	30	30	4	82	50	35	34	80	5	64	59	46
Pseudomonad B	10	12	15	-	-	-	24	-	47	-	25	-
Xanthomonad	18	-	-	-	-	-	13	10	19	-	-	-
Agrobacterium	-	-	-	18	-	65	-	-	-	-	-	-
Achromobacter	20	36	32	-	-	-	-	-	24	12	-	36
Cytophaga	2	22	13	-	10	-	29	10	5	24	16	18
Chromobacterium	20	-	36	-	40	-	-	-	-	-	-	-

C = control soil sample:counts x 10^{-6} per g.

R = rhizosphere sample:counts x 10^{-6} per complete root system.

Table 19. The nutritional classification of bacteria isolated from the rhizosphere of 6 field crops.

Adequate source of nitrogen	SAMPLE											
	Soil 7		Soil 8		Soil 9		Soil 10		Soil 11		Soil 12	
	C	R	C	R	C	R	C	R	C	R	C	R
	%											
Mineral	50	30	40	100	100	100	63	80	10	88	72	64
Amino acid	32	18	16	-	-	-	37	20	25	12	16	-
Yeast extract	18	52	44	-	-	-	-	-	65	-	12	36

Of particular interest was the reduction in the proportion of Gram-negative organisms in the rhizosphere of plants grown in soils which initially harboured a high proportion. This result, which was also obtained in Experiment 7, indicated that plant roots do not necessarily preferentially promote the growth of Gram-negative organisms. Whether or not the growth of Gram-negative organisms is promoted appeared to depend on the composition of the original soil microflora.

The results in Table 18 showed that Pseudomonas and Cytophaga strains occurred more regularly in rhizosphere samples than the other groups of bacteria. Previous findings in this study showed that these groups are frequent inhabitants of the rhizosphere. The irregular development of Achromobacter, Xanthomonad and Pseudomonad B strains does not however follow the trend observed with these groups in studies with Soils 12 and 13 in Experiment 7. The results obtained in the present experiment indicated that the composition of the rhizosphere microflora is not always correlated with the original soil microflora.

The nutritional classification (Table 19) substantiated previous findings in this study by showing that the growth of organisms requiring organic nitrogen was not always preferentially promoted in the rhizosphere. The promotion

of/

of growth by the potato and pure oat crops of organisms capable of growth on mineral nitrogen media suggested that non-nitrogenous energy sources were available in the root region. The wheat and mixed oat and pea crops promoted the growth of organisms requiring yeast extract. It seemed that amino acid nitrogen was not an important factor in the development of these microflora.

DISCUSSION.

Discussion

Counts of bacteria.

The total and Gram-negative bacterial counts recorded in the tables referred to the population of the complete root system, together with the attached soil. The counts were not related to units of root material since the weight or surface area of the root system was not estimated. The counts merely enabled the proportion of Gram-negative organisms in the rhizosphere to be compared with the proportions in the control soil and on the seed coat.

The occurrence of the Gram-negative groups in the rhizosphere.

The percentage incidence of the Gram-negative groups given in the tables, only indicated the composition of the rhizosphere microflora at the time of sampling. The composition of the microflora was not necessarily similar at any other stage of plant development. The occurrence of these organisms in large numbers, compared with the control soil indicated that multiplication had occurred and that the organisms were able to compete with other organisms for energy and nitrogen sources. Another factor affecting the composition of the rhizosphere microflora may have been the ability of the organisms to remain viable in the rhizosphere for extended periods after multiplication had ceased.

In/

In spite of considerable variation in the composition of the rhizosphere Gram-negative microflora, Pseudomonas strains developed more frequently than the other groups of Gram-negative bacteria. The development of strains of P Pseudomonas in the rhizosphere is correlated with their occurrence as a dominant type of Gram-negative organism in most of the control soils. Strains of Achromobacter, Cytophaga and organisms in the Pseudomonad B and Xanthomonad groups also occurred regularly in rhizosphere samples. The normally large development of these organisms, including the Pseudomonas strains, in the rhizosphere was usually correlated with their ability to compete on clover roots in a simple two-culture rhizosphere system with organisms from other Gram-negative groups. The Cytophaga strains were however unable to compete with most of the other Gram-negative groups, which only developed occasionally in the rhizosphere and usually not in large numbers. Microscopical examination of the development of pure cultures on clover roots growing on agar also usually showed a correlation with the occurrence of the different groups in the rhizosphere.

Examination at different stages of plant development of bacteria on the roots of oats, grass and clover grown in nutrient solution, did not reveal development which was common to the three types of plant or which could be related

to/

to the age of the plant. The composition of the Gram-negative bacterial microflora of three oat plants; one grown in soil, one in sand inoculated with soil and a third in nutrient solution inoculated with soil showed no similarity. The plants in the various environments appeared to be promoting the growth of different groups of Gram-negative bacteria. In these experiments neither the composition of the seed microflora nor the dominant control soil microflora were related to the composition of the rhizosphere microflora. In Experiment 7, in which the effect of the plant environment on the rhizosphere microflora was examined conflicting results were obtained. In some rhizosphere samples the percentage occurrence of the different Gram-negative groups was similar to that found in the control soil, in others it was not.

The nutritional classification of the Gram-negative rhizosphere organisms was based on the simplest nitrogen source supporting growth. The classification showed that although there was great variation, the growth of organisms requiring organic nitrogen compounds was usually preferentially promoted. Usually the growth of organisms requiring amino acid nitrogen was not promoted more than the growth of those requiring yeast extract.

The /

The selectivity of the rhizosphere.

The growth of Gram-negative bacteria was usually preferentially promoted in the rhizosphere. The exceptions where there was a reduction in the proportion of Gram-negative organisms, compared with the control soil, occurred in sandy soils in which plants were growing poorly. These soils, initially, contained a higher proportion of Gram-negative organisms than the fertile farm soils. Plants growing actively, usually promoted the growth of a larger proportion of Gram-negative bacteria than plants growing poorly. Presumably the actively growing plants were liberating larger quantities of organic substances than those growing poorly. Large variation was observed in the percentage occurrence of the various Gram-negative groups found in the different rhizosphere samples. There appeared to be a general selective effect on the Gram-negative organisms, rather than the selection of specific groups. The ability to utilise non-nitrogenous plant acids and amino acids as energy sources were the only detected properties which the organisms possessed in common which could account for their occurrence in large numbers in the rhizosphere. None of the organisms appeared to be specific for one type of plant or soil. The dominant Gram-negative rhizosphere bacteria did not seem to differ from the dominant organisms isolated/

isolated in a previous study (Holding 1954), from soil to which peptone had been added.

The importance of the Gram-negative bacteria.

Only on one occasion were the Gram-negative organisms observed to outnumber the remainder of the micro-organisms. The proportion of Gram-negative bacteria in 47 rhizosphere samples varied from 4 - 63% with an average approximately 23%. The average proportion in the control soils was about 13%. It can be assumed therefore that the Gram-negative bacteria were usually a numerically important group. The results obtained in Experiment 4 indicated that pure cultures of the predominant Gram-negative rhizosphere bacteria have little effect on the growth of clover plants growing in either a complete or in a deficient mineral medium.

Organic substances inhibitory to plant growth under sterile conditions were oxidised by most of the organisms tested; their detrimental effect being eliminated.

Future work on the subject.

Further studies on the development in the rhizosphere of pure and simple mixed cultures of rhizosphere and control soil organisms might enable more information to be obtained on the selective conditions prevailing in the rhizosphere and of the properties of the organisms which permit their development/

development in the rhizosphere. Chemical analysis of root exudate and the control of the plant environment would help to clarify the situation. Analysis of the rhizosphere microflora of a wide variety of plants in many types of soil would show whether or not the rhizosphere microflora were soil and plant specific. More refined techniques, possibly involving isotopic tracer studies with nitrogen, phosphorus and other important elements might show more clearly the effect of organisms on the growth of plants. In particular, competition by the organisms for plant nutrients and the part played by organisms in the transfer of plant nutrients from the soil to the plant appear to be interesting problems.

SUMMARY

Summary

Intense microbiological activity is normally found in the soil zone in the immediate vicinity of plant roots. This investigation was carried out with the object of obtaining more information about certain aspects of the so-called "rhizosphere effect", which had been largely neglected previously. Particular attention was given to the Gram-negative organisms, which had been reported to develop more than the other groups of bacteria, in the vicinity of plant roots. Consideration was given to the classification and occurrence in the rhizosphere of the Gram-negative organisms, their effect on plant growth and the reasons for their development in the rhizosphere. The effect of the environment in which the plants were grown on the rhizosphere microflora was also examined. Oats, clover and grass plants were used in the experiments.

The growth of Gram-negative organisms was usually preferentially promoted in the rhizosphere, except by plants growing poorly in sandy soils. In 47 rhizosphere samples the proportion of Gram-negative organisms varied from 4 - 63% with an average approximately 23%. The average proportion in the control soils was about 13%.

The Gram-negative organisms were classified into 13 groups on the basis of their biochemical and physiological properties./

properties. The ability to utilise non-nitrogenous plant acids and amino acids as energy sources were the only detected properties which the organisms possessed in common that could account for their occurrence in large numbers in the rhizosphere. In some rhizosphere samples the microflora was similar in composition to the dominant control soil microflora. The seed microflora did not appear to be important in determining the composition of the rhizosphere microflora. Large variation was observed in the percentage occurrence of the various Gram-negative groups found in the different rhizosphere samples. There appeared to be a general selective effect on the Gram-negative organisms rather than the selection of specific groups. None of the rhizosphere organisms appeared to be specific for one type of soil or plant. The dominant Gram-negative rhizosphere organisms did not seem to differ from the dominant organisms isolated, in a previous study, from soil to which peptone had been added. Organisms placed in the genera Pseudomonas, Achromobacter and Cytophaga and the Xanthomonad group, together with pseudomonads which appeared to be unable to oxidise carbohydrates (Pseudomonad B group) occurred frequently in rhizosphere samples. Strains in the genera Agrobacterium, Flavobacterium, Alcaligenes, Chromobacterium, the facultative anaerobes and pseudomonads requiring organic nitrogen/

nitrogen sources (Pseudomonad A group) only occurred occasionally and usually in small proportions.

The ability of Gram-negative rhizosphere organisms to compete on clover roots in a simple two-culture rhizosphere system with cultures from other Gram-negative groups was usually correlated with their occurrence in the rhizosphere. A microscopical examination was made of the development of pure cultures of rhizosphere organisms on clover roots growing on agar. The root hairs promoted a greater development of organisms than the other parts of the root system and were presumably liberating larger amounts of organic substances.

The Gram-negative organisms were also classified nutritionally on the basis of the simplest nitrogen source supporting growth. The organisms were examined for their ability to grow in media containing either a) mineral nitrogen, or b) amino acids, or c) yeast extract. The classification showed that oat and clover plants growing actively usually preferentially promoted the growth of organisms requiring organic nitrogen. The growth of organisms requiring amino acid nitrogen was not normally promoted more than the growth of those requiring yeast extract. Usually oat plants growing poorly and grass plants promoted the growth of organisms requiring only mineral nitrogen sources.

Examination/

Examination at different stages of plant development of bacteria on the roots of oats, grass and clover grown in nutrient solution did not reveal development which was similar for the three types of plant or which could be related to the age of the plants. The composition of the Gram-negative rhizosphere microflora of three oat plants; one grown in soil, a second in sand inoculated with soil and a third in nutrient solution inoculated with soil, showed no similarity. The microflora of the nutrient solution was not the same as that of the roots growing in the nutrient solution.

Oat plants were grown in three different soils in five environments. The different environments were created by altering the light intensity and temperature, by applying fertilizer and by growing the plants in nutrient solution. Differences in the composition of the rhizosphere microflora, associated with the environments, were demonstrated, but no one difference was shown in all the soils.

Pure cultures of bacteria exerted only a slight effect on clover plants growing in sand-nutrient solution media. The severity of nitrogen, phosphate and potassium deficiency symptoms shown by sterile plants was not increased by inoculation except by three organisms inoculated into the phosphate deficient medium. The addition of glucose, sodium malate/

malate or Casamino acids to either sterile or inoculated plants did not bring about improved plant growth. The toxic effect of certain concentrations of these substances on sterile plants was not usually observed with the inoculated plants.

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